

Should courts decide on euthanasia?

By making a sick and malformed child a ward of court, three British judges have drawn attention to the need for a judicial procedure in cases where sick people seek euthanasia voluntarily.

EUTHANASIA is a perpetually haunting issue that usually arises in relation to the elderly sick or adults who are terminally ill. The argument that people in such a plight should be enabled by their physicians to die is a powerful one, at least when the demand is a voluntary decision of the person concerned, and when there are medical grounds for believing that he or she is in any case nearing death. Why needlessly prolong suffering? The difficulty, for the supporters of voluntary euthanasia, is that the counter-arguments are also strong — stronger than the euthanasia lobby (every country has one) acknowledges. Knowing whether an expression of a wish to be allowed to die is strictly voluntary is one difficulty; telling the difference between a wish to die and a wish not to be a nuisance to others can never be simple, for example. And (*pace* current practice in The Netherlands) there is force in the argument that legitimizing physician-assisted suicide would be a dangerous precedent and invidious for physicians.

Now the British courts have been thrust into a situation of this kind, and have responded in an enlightened fashion that may point the way to a more generally accepted policy on euthanasia. The case on which three Law Lords adjudicated last week concerns a child born more than a year ago with severe malformations. The extent of the brain damage (suffered *in utero*) is apparently so great that the child will never be mentally competent. Although not dependent on an incubator for survival, the child must be fed through a tube inserted into its stomach. Its time is divided between a hospital and its parents' house, who care for it. The parents had applied to the courts for the termination of the child's life, presumably by starvation.

This is not a case of voluntary euthanasia, of course; the child has no say in the matter. The case for ending the child's life is partly to lift the burden of distress and care imposed on the parents and, less cogently, partly because the child has no future as a person. Religious people will strongly object on both grounds, and their objections cannot be lightly brushed aside. Freedom from distress is not an absolute right, but is (or at least can be) ennobling. And who is to say that a person whose IQ is not measurable is not a person? A more immediate difficulty for the courts would be that granting this application would presumably stimulate a flood of similar applications by the relatives of elderly and incompetent persons. So what did the court decide? Make the child a "ward of court", which is a procedure in which the responsibilities of parenthood pass to the

legal system. In this case, the child will continue to live with its parents and in their local hospital. The court has said that it will hear further argument from the parents when they have more to say.

That decision is enlightened because it makes a decision about life or death a judicial process, which is right and proper. Why should not the same procedures be followed in cases of voluntary euthanasia? Then it would be possible to test by taking evidence whether a person's expressed wish to die is strictly voluntary and also to ensure that the medical circumstances of the case are generally agreed. Distressing though they would be, cases of this kind should be heard in public, for death (like birth) should be a matter of the public record. But the chief benefit of such a development is that it would rid physicians faced with demands to be helped to die of a responsibility that should not be shouldered individually. □

Privatization goes wrong

Britain, with a recent rich record of denationalization, has error as well as success to boast of.

NOT for nothing has the British government been boasting over the past decade that its programme of selling off nationalized industries to would-be private owners has been widely emulated elsewhere in the world. Governments of all stripes have followed suit. Not only the free-market economies of Western Europe, but the states of Central Europe and the ex-Soviet Union, have joined in the public auction of what were previously assets of the state. The Russian government in the past two years has surprised its voters and even itself by the speed and the degree to which it has turned over the ownership of state industry to the general public.

In all cases, the motives behind the drive for privatization appear to be similar. Governments have discovered that the means by which politicians and civil servants are recruited to the public service do not ensure their aptitude as industrial managers, that it is often a waste of financial resources that public capital should be locked up in enterprises that private persons would be willing to own and that there are intolerable conflicts between the interests of nationalized industries (especially when they are in trouble) and the public interest generally. For all those reasons, the fashion for private



rather than public ownership will spread, and has probably come to stay.

None of that implies that all privatization is virtuous and deserves to be applauded. The British government's own record in the field, although admirably innovative, has been patchy in its execution. The impending much-delayed privatization of the nationalized railway system has all the makings of disaster, not only because of the complexity of the administrative arrangements decreed, but because many parts of the system will require continuing public subsidies when they are in private hands, raising the prospect that the government will be perpetually locked in argument over the level of subsidy with rail operators who will easily recruit in their aid all those likely to be harmed or inconvenienced by depleted services. That is a recipe for perpetual electoral unpopularity, for the present government and its successors.

Meanwhile, some of the British government's past privatizations have begun to unravel at the edges. The most obvious case is that of the electricity industry, most of which was sold off just over five years ago. Previously, Great Britain (the United Kingdom less Northern Ireland) had been supplied with electricity by three generating boards (two in Scotland) and by a dozen or so distribution boards organised on a regional basis. At privatization, the regional boards became separate companies (now called 'recs') and were given power to generate their own electricity if they wished. The generating board outside Scotland was split into three parts, one of which (called Nuclear Electric plc) owns nuclear power stations almost exclusively and will be privatized next year (if nothing goes wrong).

Curiously, the recs were made joint owners of what used to be called the National Grid, a high-voltage distribution system that makes it possible for a small country such as Britain to make economical use of bulk electricity generation. At the time, the theory was that the recs were likely to be at the mercy of the generating companies, so that effective control of the grid would give them a chance to answer back. But events have predictably shown that the shoe is on the other foot. The rules give the recs an incentive to meet their region's base-load for electricity from power stations they build themselves and to look to the generating companies only for the daily peak demand for electricity. So far, the threat that that might happen has ensured that it has not done so.

The cat that has now been set among this flock of pigeons is the expiry of the government's "golden share", effectively gave it the right to veto changes of ownership of the electricity companies. The first sign of trouble was a bid for a company called Northern Electric by a conglomerate called Trafalgar House, with no expertise in electricity generation or distribution; that bid has been turned away by the rec's decision to return to its shareholders the equivalent of what they had paid for their shares in the first place. As a result, the official regulator of the industry decided to toughen the conditions that the power companies must satisfy in the years ahead. But that has not been enough to deter other bidders for other companies; one rec (Eastern Electric) has been snapped up by Hanson plc for £2.2 billion, another is

under siege by a US electricity utility.

All this is predictable and should have been predicted. Left to themselves, the recs would now presumably recombine into the monopoly they were before privatization. Somewhere along the line, the government would put a stop to that with a reference to the Monopolies and Mergers Commission. But to those with deep pockets, a rec is a good buy because only modest skill in exploiting technical innovation should make it easy to beat the inflation-linked price caps which are the standard regulatory technique in Britain, while the largely unexploited self-generation of electricity offers a rec the opportunity to become a major generating company as well. In the long run, the result may be genuine competition among a few recs to be a major generator of electricity as well, but the process will take time. It seems a roundabout way to reach a goal that might have been specified at the outset. The trouble with privatization is that it is, in practice, irrevocable. □

Festival of chips?

The British Association's visit to Newcastle next week could be good for both.

THE British Association (for the Advancement of Science) is a venerable institution in the strict sense of that word. Founded in 1835, it functioned as a lively and informal scientific society during much of the nineteenth century. But for at least the past half century, it has not been that at all. The annual meeting is no longer what it was at the beginning of this century, a forum at which people with original discoveries to report would argue their significance in public, and has become what the programme for this year's meeting (at Newcastle from 9 to 15 September) calls itself — a "festival of science". There is no shame in the changed role of the annual meeting. The profession of science has become more professional than formerly, and who will say that the present time does not need a regular occasion when a wider circle of people can celebrate what science can do well?

Sadly, the BA has not always lived up to this expectation. The sense of festival has too often been undermined by the homespun character of too many of the presentations, by spartan or at least unfestive surroundings and by general aimlessness. In the recent past, the BA has found itself holding its meetings at universities that are not conspicuously centres of population, with the result that excellently constructed programmes have been poorly attended. Mercifully, this year may change that. Newcastle is not merely a conurbation as well as a city, but it is also still hugging itself in the knowledge that Siemens, the German manufacturer, intends to build a major microchip plant in the region. After miserable decades during which shipbuilding and coalmining in northeast England have drifted into oblivion, the Tyneside region has a sense of festivity again — and a practical need to learn about microchips. Would it not be grand if some of that rubbed off on the BA? Like Newcastle, all it needs for survival is a little luck. □

Clinton's science adviser embroiled in 'book-burning' row over risk bill

Washington. In an unprecedented move that reflects the increasing politicization of US science policy, the chairman of the House of Representatives Science committee has complained to President Bill Clinton that his science adviser, Jack Gibbons, has been engaged in "mudslinging and name-calling", and has asked the president to take appropriate action.

The complaint has come from Robert Walker (Republican, Pennsylvania), in a letter written to Clinton on 17 July. It was prompted by a press briefing on environmental research at which the normally mild-mannered Gibbons described Republican proposals for including more explicit risk assessment in environmental legislation (see *Nature* 373, 180; 1995) as "the scientific equivalent of book-burning".

Walker is a major force behind the risk assessment proposals, which are at present stalled in the Senate. In his letter, Walker

wrote that "in recent weeks, certain officials have gone well beyond the bounds of legitimate policy debate and have entered into a particularly despicable type of mud-slinging and name-calling".

Walker adds: "the only 'book-burners' with which I am familiar in recent world history were the Nazi Party fanatics of Hitler's Germany". He describes the equating of "those who have a legitimate political difference with your administration" with Nazis as both "personally highly offensive and totally unacceptable".

He concludes: "I trust you will take

immediate steps to ensure that all members of the White House staff heed your own ground rules on 'civility', and that those responsible for such unseemly public statements are appropriately counselled."

The letter appears to mark a new low in the deteriorating relations between Democrats and Republicans on science policy, an area once relatively immune from partisan politics. Democrats who have seen the letter accuse Walker of hypocrisy; with his close friend Newt Gingrich, now the leader of the House, Walker developed a fearsome reputation in the previous Congress for his mastery of techniques of political manipulation.

But the letter, which has not been released by either Walker's staff or the White House, does not seem to have been a publicity stunt. Walker is known to have been angered by several of Gibbons' pronouncements this year, in particular a highly partisan speech delivered to a packed policy seminar held by the American Association for the Advancement of Science (AAAS) in April (see *Nature* 374, 667; 1995).

Gibbons' line on book-burning isn't even a fresh one: it was used last February, just as provocatively but less surprisingly, by Bruce Babbitt, the Secretary of the Interior, in a combative speech on Republican plans to close the National Biological Service (see *Nature* 373, 646; 1995).

Many observers have been startled at Gibbons' recent partisan tone. Some speculate that he is deeply angered by Republican moves to shut down the Office of Technology Assessment (OTA), of which he was director for 13 years before he moved to the White House in 1993. Protocol has prevented Gibbons from defending OTA, which is an office of Congress.

Gibbons' job as both Clinton's science adviser and director of the White House Office of Science and Technology Policy has changed drastically since Republicans took control of Congress last November. Before then, he was helping to run the country, coordinating science policy through innumerable back-room meetings. Now young, clean-cut Republican congressional staff are running the country, and Gibbons has had to join Walker and Gingrich in the political bear-pit, fighting to put over sound-bites.

A spokeswoman for Walker says that he and Gibbons still have "very friendly personal conversations", and that Walker's objection was to the science adviser's "public rhetoric". The normally communicative White House press office did not return calls on the subject, possibly indicating a reluctance to engage in further public squabbling with Walker. **Colin Macilwain**



Walker: asking Clinton to ensure 'civility'.

Grants continue despite planned closure

Washington. The US National Institute of Standards and Technology (NIST) plans to announce a series of new awards under its Advanced Technology Program (ATP) within the next few weeks — despite the wish of Congress to close down the controversial programme.

NIST will grant eight fresh batches of ATP awards between now and September, most of three years' duration. According to Brian Belanger, deputy director of the programme, it will do this in order to "fulfil its obligation" to industrial partners that have already invested time and money in preparing proposals.

But Republicans in the House of Representatives, who are proposing that the ATP be closed in October, are furious. "NIST is making a terrible mistake by spreading money out on new contracts," says Robert Walker (Republican, Pennsylvania), chairman of the House Science committee.

NIST is required to meet commitments under its contracts only if the money to do so is duly appropriated by Congress. Walker predicts that NIST, having started these contracts, will now come back to Congress next year, pleading for money to finish them.

But he describes NIST's granting of new contracts as "a horrible decision" that will cost the agency support in Congress. "They ought to be reserving money so they can complete the

contracts they [have] already [signed]."

Last month, NIST awarded grants to 17 projects for new technologies in all fields, and another seven to help develop tools for DNA analysis, at a total cost of \$61 million. By September, the agency will announce the winners of competitions for grants in another ten specified fields, ranging from catalysis technology to digital data storage; these involve about \$150 million of NIST investment in the first year alone.

Even after a \$90-million cut in a rescission bill signed by President Bill Clinton last month, NIST still has \$341 million to spend on ATP in the financial year which ends October. Clinton has requested \$491 million for the programme next year; the House appropriations committee wants to cut out all of this, while the Senate will put forward its own figure after the August recess.

The conflict between the Democrat administration and the Republican Congress over the ATP is one of an array of policy and budget issues that will be finally resolved in October, when the two sides must agree on a budget bill. The granting of new ATP money is intended to indicate the administration's determination to maintain the programme.

But Clinton did not block cuts to the ATP when he made compromises last April, and some of the its keenest supporters doubt he will fight for it. **C. M.**

Brazil examines claim that research is losing out in science funding

São Luís, Brazil. Brazil's Ministry for Science and Technology has launched a review of the effectiveness of its expensive postgraduate scholarship funding programme, which some claim is distorting science funding.

According to Jose Galizia Tundisi, the new president of the National Council for Scientific and Technological Development (CNPq), the agency spends about 380 million reais (\$340 million) a year on scholarships — but only about 10 per cent of this figure on research grants. (CNPq is the ministry of science and technology's main funding agency.)

Tundisi told the annual meeting of the Brazilian Society for the Progress of Science (SBPC) in São Luís last month that he accepts that the country needs 100,000 active scientists. But he expressed concern about the quality of the research that they may be able to produce. "CNPq pays about 44,000 scholarships; I need to know what they are doing, and what sort of science the country is getting," he said.

The government is already supporting studies of scientific output, and a new programme — known as System for the Evaluation of Scholarships Abroad or SABE, the Portuguese word for 'knows' — is now trying to measure how much Brazilian students and scientists have benefited from time spent abroad. According to Tundisi, the first results, based on interviews with one hundred returning scientists, show Brazil needs to take action to avoid duplication of research efforts.

The decision to pour resources into postgraduate education and training was made by the government of President José Sarney, which held office between 1985 and 1990. According to Crodowaldo Pavan, who headed CNPq during that period, the move was a deliberate effort to boost the country's scientific capabilities; he told the SBPC meeting that it is now up to Sarney's successors to find resources to keep Brazil's newly qualified scientists usefully occupied.

Pavan said politicians told him he should first obtain the money for scholarships in order to attract people into science, and that the money for research itself would then follow. "As a result, we have given more scholarships in three years than in the previous 31 years of CNPq's existence", says Pavan. But now the bill has arrived.

Lindolpho de Carvalho Dias, executive secretary at the ministry, says that efforts are now being made to correct these numbers. But he points to at least one advantage of the scheme, namely that "all good students are now receiving scholarships". Ricardo Bonalume Neto

Synchrotron proposals spark high-energy debate in Japan

Tokyo. A proposal by Tokyo University's Institute for Nuclear Study (INS) to build a powerful 50-GeV proton synchrotron has split Japan's high-energy physics community. While some leading high-energy physicists support the proposed facility, others fear that it could undermine their plans to build the world's next giant linear collider.

The synchrotron would be a key facility at a planned new complex of three national research institutes in Tsukuba science city, north-east of Tokyo. The three institutes — for high-energy physics, nuclear studies and synchrotron research — are expected to be set up in 1997 through reorganization of the National Laboratory for High Energy Physics (KEK) in Tsukuba, and the relocation of INS and its conversion to a national research institute, like KEK.

The proposed Japan Linear Collider (JLC) would consist of two linear accelerators (linacs), with a combined length of 25 km, for colliding beams of electrons and positrons, each with energies of 150–250 GeV (see *Nature* 370, 169; 1994). Considerable research and development on the JLC has already been carried out at KEK.

The synchrotron proposal caught the high-energy physics community by surprise when it was announced by INS about two months ago, setting off a surge of protest by electronic mail. The idea was not entirely new, as INS has been discussing for about ten years its proposed Japan Hadron Project, which originally called for a 1-GeV proton linear accelerator, but included the possibility of adding a high-energy synchrotron in a second phase (see *Nature* 353, 200; 1991). But the idea of starting with a powerful (and expensive) synchrotron is new.

Current plans for the synchrotron facility feature a 200-MeV linac and a 3-GeV booster ring, from which a small fraction of the beam will be tapped off to the main 50-GeV ring. Sakue Yamada, director of INS, says the facility's cost is estimated at ¥75 billion (US\$830 million). This is considerably more than the original plan, and supporters of the JLC fear it will make it more difficult for them to obtain the ¥200 billion-plus needed for their own machine.

A meeting was held about a month ago between leading nuclear and high-energy physicists to try to calm the situation. Yamada says that the agitation among high-energy physicists "has cooled down", and hopes that supporters of the JLC accept that the synchrotron is not a threat to the linear collider, as the much larger budget for the collider would have to come from a different budget category. One JLC proponent rejects this argument. He says the general public is not aware of different budget categories, as

it is "all the same tax" that pays for such facilities and the public "will probably ask why scientists need two such machines". But Yamada points out that, compared to high-energy physics, both nuclear and solid-state physics have received little government funding in recent years. He points out that the proposed synchrotron facility could be used by solid-state physicists and biologists as well as nuclear and high-energy physicists.

Apart from competition for funding within the physics community, a long-standing rivalry between the Ministry of Education, Science and Culture (Monbusho), which funds both INS and KEK, and the Science and Technology Agency (STA), has helped to launch the INS proposal. Ten years ago, the STA upset both Monbusho and the ministry's synchrotron researchers by deciding to build the world's largest electron synchrotron, the 8-GeV SPring-8, which will be completed in a few years time in Nishi Harima, west of Osaka. In the process the STA "took some power away" from Monbusho, says one physicist at Tokyo University, and the education ministry is now seeking to regain its position with the INS proposal.

The STA is planning to build a very high-powered 1.5-GeV proton linac as a neutron source and has the ultimate goal of using the proton beam to dispose of nuclear waste. Some efforts were made by researchers to coordinate this with the earlier — and less ambitious — Japan Hadron Project. But the talks did not progress far, and Monbusho and INS chose the more ambitious path of a 50-GeV proton synchrotron in part to distinguish themselves from the STA project.

INS's proposal is to use the existing infrastructure of a 12-GeV proton synchrotron at KEK, which will be replaced by the 50-GeV machine. The new synchrotron will be accommodated in a 1.4-km circular tunnel within the main ring of KEK's TRISTAN positron-electron collider.

The 3-GeV booster will be used as a source of pulsed neutrons, muons and exotic nuclei for experiments to be carried out mainly by solid-state physicists. The 50-GeV synchrotron will also produce high-intensity meson/neutrino beams, and will be used to extend intermediate energy nuclear and particle physics by, for example, carrying out very-fine-resolution experiments on hyper nuclei, hyperon-nucleon scattering, heavy ion reactions and kaon rare decays.

A special open meeting for high-energy physicists will be held next month to discuss the INS proposal and the JLC. According to Yamada, nuclear physicists will also participate and they hope to achieve "better understanding" between the two communities.

David Swinbanks

Go-ahead for tree salvage angers US Greens

Washington. Large-scale logging is set to resume in the United States after President Clinton signed a budget rescission bill which includes language to exempt so-called salvage logging from environmental laws.

The forestry industry has argued for the exemption on the grounds that burnt and otherwise damaged trees should be removed in the interests of better forest maintenance. But environmentalists regard the measure as a 'front' for the resumption of extensive logging, and are livid with President Clinton for allowing its passage.

The measure is the first of the environmental changes proposed by the Republican Congress to actually pass into law, and environmental pressure groups see its signing by Clinton on 27 July as an indication that he is not prepared to confront Congress on environmental issues.

"This is a betrayal of everything Bill Clinton said he stood for," says Carl Pope, executive director of the Sierra Club, the largest environmental pressure group in the United States. Pope claims that the administration has come to a private agreement with Congressional leaders to use the new law to per-

mit twice as much logging as scientists at the US Forest Service believe to be necessary.

The law essentially says that tree-clearing on public land which can be classified as "salvage logging" will be permitted by federal agencies during a year-long "emergency period", regardless of existing federal laws and international agreements.

Most ecologists agree that selective logging can be beneficial to forest management in some circumstances (see *Nature* 370, 585; 1994), but the nature of these circumstances is hotly disputed. Environmentalists charge that the Forest Service has used salvage logging as a pretext for logging the largest trees — the only ones still alive — after forest fires.

A spokesman for the Forest Service said it could not say how it would implement the new law until it had been advised by lawyers at its parent, the Department of Agriculture.

In an ambiguously-worded memorandum to the department heads involved, Clinton

Logging on: Salvage logging will be legal for one year.

IMAGE
UNAMAGABLE
FOR A CORP
FOR A BIG
FOR A RIGHT
REASONS

said he "did not support" the timber salvage provisions in the law he had just signed. The memo nevertheless instructed the agencies "to implement these provisions in an environmentally sound manner."

The memo disappointed environmental groups, who said they planned court action to block salvage logging authorized under the new law. "We will be going to court," warned Pope. "This is an attempt to suspend the rule of law".

Colin Macilwain

Daniel Dancer/Still Pictures

Medical research charities 'can rely on peer review'

London. Britain's Charity Commissioners have backed away from an earlier proposal that the trustees of medical research charities should be held directly responsible for checking on the scientific quality of research published by their grant recipients. They have also dropped a parallel suggestion that charities be required to lay legal claim to any potential patents resulting from such research.

In draft guidelines on the conduct of research charities published in London last week, the commissioners suggest that trustees can rely on the peer review process of a "reputable scientific or medical journal" in which the results are published — although they add that, if further publicity is to be given to these results by the charity concerned, its trustees should take additional steps to satisfy themselves that the results are soundly based.

As far as intellectual property rights are concerned, the commissioners propose that all charities should be responsible for ensuring that these are adequately protected, as part of their broader responsibility for their public dissemination. But they suggest leaving open the question of whether the charity itself should file for such protection — as an earlier draft would have required — or whether this can be left to the institution at which the research is carried out.

The new guidelines have been welcomed by the Association of Medical Research

Charities (AMRC), which feared that earlier draft proposals on the monitoring and dissemination of research were too restrictive. "We are very pleased that [the proposed guidelines have] come out like this," says Diana Garnham, general secretary of the AMRC.

The responsibilities of medical charities for the impact of the research they sponsor were dramatically highlighted in 1990 by the publication of the preliminary results of a study, backed by two cancer research charities, of the activities of an alternative cancer therapy centre in Bristol.

The study, published in *The Lancet*, claimed that the survival rate of those treated at the alternative centre was substantially lower than that of those receiving conventional treatment. But these conclusions were subsequently withdrawn when it was revealed that they had been based on faulty statistical analysis.

Last year, following a lengthy internal discussion of the implications of the way in which the preliminary results of the Bristol study had been published, the Charity Commissioners published various draft recommendations, including the proposal that the trustees of a charity should be responsible for "the evaluation of the products of research they have funded before the results are published".

This immediately produced the criticism that neither trustees nor the institutions in which the research is carried out are often

in a position to assess the intellectual quality of research results, and that this is best handled by the conventional peer review process, in particular that under which scientific and medical journals operate (see *Nature* 367, 100; 1995).

This conclusion now appears to have been accepted by the commissioners. Their latest proposals, which are being circulated for comment, confirm that while charity trustees must accept responsibility for the proper evaluation of the results of research before it is published, "if the results are formally published in a reputable scientific or medical journal, trustees may rely on an evaluation of quality by the journal concerned".

The proposed guidelines on the handling of intellectual property are aimed primarily at larger charities, such as the Wellcome Trust. These have been seeking clarification of the extent to which their desire to become directly involved in the exploitation of the results of research that they sponsor could conflict with their charitable status.

But the proposed guidelines have also come as a relief to some smaller medical charities. These had been concerned at the costs which would have been involved if, as earlier suggested, they had been required to seek legal protection on all patentable discoveries. "We just do not have either the resources of the staff to do that type of thing," says Martin Scott, research director of the Cystic Fibrosis Trust. David Dickson

Fertility pioneers face 'misconduct' charges

San Diego. A world-renowned group of fertility specialists at the University of California (UC) at Irvine is being investigated for scientific misconduct, including the charge that members performed research on patients without their knowledge or consent.

Other allegations being investigated by federal and state agencies are that they used human oocytes said to have been discarded for research, also without consent, and smuggled into the United States an unapproved drug that was then sold to women patients to stimulate the production of eggs.

Two of the physicians involved — Ricardo H. Asch and Jose P. Balmaceda — are known internationally for developing in 1984 a technique known as gamete intrafallopian transfer (GIFT) while at the University of Texas at San Antonio. Asch is originally from Argentina, while Balmaceda and the third physician, Sergio C. Stone, are from Chile.

The allegations of misappropriation of human eggs and embryos centre on Asch. University officials say medical records indicate that eggs or embryos of at least 35 women may have been used without their consent, and that at least six children were born to unsuspecting parents as a result of the procedures used.

In one case, according to the records, eggs were taken from a young woman undergoing a fertility procedure, mixed without her knowledge with the sperm of a man, and the resulting fertilized embryo implanted in the man's wife, who had suffered a premature ovarian failure. A former administrator of the fertility clinic, Debra Krahel, who discovered some of the alleged irregularities before being forced by the university to resign last year, has called this practice "biomedical rape".

None of the three physicians is prepared to discuss the allegations with the press, but all have, through their lawyers, denied any impropriety. Asch blames some of the problems on poor record-keeping, and has claimed on occasion that allegations that embryos were misappropriated originated from an attempt to extort money from him.

The physician's lawyers acknowledge

some research was carried out without the required approval of the UC Irvine human subjects committee. But they claim this resulted from a misunderstanding of university policies. At least six research articles were published from this research, involving three retrospective studies and three studies involving drug trials or embryo-freezing.

According to the report of an investigation carried out by the university, Asch has acknowledged dispensing the unapproved hormone medication, HMG Massone. But both Balmaceda and Stone have contended in public statements that they have been smeared by university officials who, they claim, have been carrying out an unfair investigation based on what Balmaceda, at a hearing before state legislators, has described as "untested allegations".

Early in June, university officials closed down the physicians' primary clinic, the Centre for Reproductive Health, which was adjacent to UC Irvine Medical Center in Orange, California, and Asch resigned from the hospital's medical staff.

All three physicians were placed on paid administrative leave from their faculty appointments at the university, while the University of California at San Diego (UCSD), at which Asch operated a 'satellite' fertility programme, did not renew his unpaid appointment.

A subsequent audit of 155 patients' records at UCSD, the results of which were released two weeks ago, revealed that the eggs of three patients may have been used in donations to two other patients with proper consent. In addition, university officials found 17 cases in which egg/embryo records were either unclear or incomplete, and that records on the retrieval of eggs may be missing for up to 50 patients.

"We are determined to learn the truth regarding any improprieties, breach of ethics or violation of patient trust," says Thomas Moore, acting chief of reproductive medicine at UCSD.

But the researchers are not the only ones under fire. After details of the scandal became public in May, there was harsh criticism of UC Irvine officials, who were

accused of having moved too slowly and of silencing whistleblowers with hush money.

Last year, when three administrators — including Krahel — attempted to investigate some allegations against the physicians, they were forced by the university to resign, awarded more than \$900,000 in settlements, and told not to publicize the case.

This move has prompted outrage from some state officials, including Lieutenant Governor Gray Davis. "It is absolutely unac-



ceptable for this publicly funded university to throw this kind of hush money around," Davis has said.

But Sidney H. Golub, executive vice-chancellor of UC Irvine and himself a physician, denies such allegations. "The university administration responded to accusations of misconduct, no matter how sensitive the issue, with thorough investigations," he says. "Protecting the privacy of the patients, getting to the truth and ensuring due process have been and continue to be our main objectives."

Despite Golub's reassurances, the university decided in late June, in an administrative shake-up prompted by the debacle over the fertility clinic, to dismiss the medical centre's senior executive, Mary Piccione. She is now contesting the termination of her contract, contending it was improper.

In the wake of the affair, officials at the American Medical Association are examining professional standards for US fertility clinics, which are largely unregulated.

Meanwhile, UC Irvine nervously awaits the results of various federal and state investigations. The federal Office for Protection from Research Risks, for example, is looking into the allegations about research techniques, while the Food and Drug Administration is investigating the allegations that unapproved drugs were used.

University officials are also examining allegations the physicians may have siphoned off tens of thousands of dollars paid in cash for the fertility treatment, not usually covered by health insurance. **Rex Dalton**

Report criticizes costly industry LINK scheme

London. A British government scheme to promote research partnerships between industry and universities has been criticized in a report published last week by the National Audit Office (NAO).

The LINK initiative, one of eight such schemes intended to support innovation and industrial competitiveness, has been described as costly and subject to unnecessary administrative delays, according to the report on all eight schemes.

LINK has supported 570 projects since

its launch in 1986. But, according to the NAO report, more than half of the grants allocated, constitute "deadweight expenditure" — funding in excess of the minimum needed to ensure a project proceeded.

Peter Dukes of the Medical Research Council, which announced the launch of two LINK research programmes on the same day as the NAO report, pointed out, however, that an earlier internal review of LINK had already identified areas that needed improvement. **E. M.**

Cash limits curb Max Planck Society's plans in the east

Munich. Plans to set up a full complement of Max Planck research institutes in east Germany by the end of the century are likely to be delayed because of a lack of funds. The Max Planck Society (MPS) says this is because the federal government's promise of a five per cent annual grant increase will, in practice, be worth only half that amount.

The MPS has already created ten research institutes in the new *Länder*, and had been hoping to increase this to between 15 and 18 by 2000, an equivalent research strength within the Max Planck system to that of the old *Länder* of west Germany.

This goal of regional balance represents something of a compromise for the research organization, which usually rejects demands for *juste retour* — the idea that benefits should be divided between sponsors according to the size of their contribution.

Although its funding is shared equally between the federal and *Länder* governments, the MPS normally argues for the right to establish research institutes wherever the appropriate supporting academic strengths — for example, in university departments — already exist. But an exception was made after reunification because of the need to establish a strong research environment in the new *Länder*.

The MPS was initially criticized for its apparent slowness in establishing itself in the east. As its research activities are distributed over the whole of Germany, it was reluctant merely to take over institutes of the former East German Academy of Sciences — only two of the new institutes are based on research being carried out prior to reunification. Instead, it picked new research areas to complement its overall activities, and then, after taking geographical balance into account, reached agreement with local universities and other research establishments in east Germany, to build up their research strengths in these areas.

But this plan depends on a renewal of the so-called 'five-by-five' agreement under which both the Max Planck Society and the grant-giving agency, the Deutsche Forschungsgemeinschaft (DFG), were guaranteed a five per cent increase in funding for each of the five years 1991 to 1995. But the government, faced with economic recession, has been reluctant to agree to this.

The federal finance ministry has agreed with Jürgen Rüttgers, the research minister, on a five per cent increase for both organizations next year, and to plan for the same increase for the following three years. This proposal will go to the *Länder* governments next month, and, if approved, to the federal parliament before the end of the year.

The DFG is pleased with this arrange-

ment. But the MPS believes that it has been short-changed. Wolfgang Hasenclever, general secretary of the MPS, claims that the government's offer to the MPS represents only a 2.5 per cent increase over its budget for the current year — and that this will leave it with DM200 million (US\$143 million) less than it needs to realize its plans.

According to Hasenclever, the difference has arisen because the government based its calculation on a budget for the MPS that excluded money allocated to the establishment of social sciences centres which will be taken over by universities next year. The MPS argues this sum belongs within their basic budget, but the research ministry says their expenses must be considered separate.

The government adds that it recognizes the MPS's need for money for new buildings. But the MPS says that this amounts at most to a noble, but as yet unfulfilled promise. The organization has not been

IMAGE UNAVAILABLE FOR COPYRIGHT REASONS

Hubert Markl, a zoologist at the University of Constance, was last week confirmed as new president of the Max Planck Society. He takes over when current president, Hans Zacher, retires from office next June. Markl has wide experience in research policy, including a term as DFG president.

offered any additional infrastructure funds next year, and although DM7 million has provisionally been included in the plan for 1997 and 1998, this will be renegotiated in the light of the situation then facing the MPS when budgets for these two years are fixed. It is also only one-third of the money that the MPS had planned for building-work during these years.

Acknowledging that Germany is one of the few countries that are actually increasing their research budgets, Hasenclever says that the MPS is accepting the situation, albeit reluctantly. Indeed, a delay in its plans may not be as serious as the MPS claims, as the organization has itself had difficulty in meeting its timetable for building up research institutes in the east where it has had recruiting problems.

Of the ten institutes already founded, only three have their full complement of scientific directors. "It has been difficult to persuade people to go [to east Germany] because the social conditions are so poor, particularly schooling", says Hasenclever. "You really need people with a pioneering spirit."

Alison Abbott

Panel clears professor involved in studies of 'close encounters'

Boston. Harvard University has decided not to take any disciplinary action against John Mack, a professor of psychiatry at the medical school who has been carrying out studies of individuals claiming to have been abducted by extraterrestrial beings.

The university announced its decision last week after the completion of a year-long investigation of the author of the best-selling book, *Abduction: Human Encounters with Aliens*. "The process is over," said Anne Taylor, counsel for the university. "John Mack is in good standing as a member of the Harvard faculty."

Until now, the very existence of a committee appointed by Daniel Tosteson, dean of the medical school, to review Mack's UFO-related research had been kept secret. Indeed, the university did not officially confirm a review panel had been set up until the inquiry had been completed and the panel disbanded.

The university has now confirmed that the faculty committee was chaired by Arnold Relman, professor emeritus at the medical school and former editor of the *New England Journal of Medicine*, and had been charged with looking into the "clinical care and clinical investigation" that Mack had carried out with those claiming to have been abducted by UFOs.

There had been concern, in particular, over whether Mack had been attempting to treat such individuals, or to study them, and over whether he had secured the formal permission needed to conduct research on human subjects.

According to Keren McGinty, a spokesperson for the medical school, Tosteson had emphasized to Mack that, in his enthusiasm to care for and study this group of individuals, "he should be careful not in any way to violate the high standards for the conduct of clinical practice and clinical investigation that have been the hallmark of this faculty".

Critics of the closed-door inquiry, some of whom had branded the proceedings a "witch hunt", had expressed fears that Mack's academic liberties were being trampled on. But according to McGinty, Tosteson confirmed that Mack was free "to study what he wishes and to state his conclusions without impediment".

At the same time, although Mack's academic freedom has been upheld, many feel that he has also been served public notice that his work must conform to stringent standards of both scholarship and medical practice. Neither Mack nor his lawyer, Eric MacLeish, has commented on the university's decision to set up a review committee, or on its outcome. Steve Nadis

International collaboration

SIR — The letter from J. Sylvan Katz and Diana Hicks (*Nature* 375, 99; 1995) underlines some of the reasons why the Burroughs Wellcome Fund, a non-profit foundation in North Carolina, United States, provides approximately \$1.3 million each year to support international and domestic collaborations among scientists.

In response to the need for scientists to exchange views with and obtain research techniques from colleagues in other countries, the Wellcome Trust in England and the Burroughs Wellcome Fund created in 1979 an exchange programme called Wellcome Research Travel Grants. Intended to advance medical science by speeding the transfer of knowledge and skills, the grants provide US and UK researchers in the health sciences with travel and subsistence support for periods ranging from 2 weeks to 6 months. More than \$100,000 in grants was awarded in 1994.

Other fund award programmes to support international and domestic collaborations include the Hitchings-Elion Fellowships, which provide US scientists early in their careers with training in the United Kingdom, and visiting professorships. They also enable researchers from the United States and elsewhere to spend time at US medical schools, universities and other non-profit research institutes. The Hitchings-Elion Fellowships enable postdoctoral fellows to work in a UK laboratory for 2 years and return to the United States for the third year of the award. More than \$900,000 was awarded in 1994 for these scientists.

The Wellcome Trust, our sister foundation and the world's largest medical charity, also provides substantial support for a variety of other programmes that foster international collaborations.

Enriqueta Bond

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Popov and Marconi

SIR — In this centenary year of wireless, Oersted G. Vendik is justified in commemorating Popov as a pioneering physicist and electrical engineer¹. Nevertheless, his portrayal of Popov's achievements, distorted by their dissociation from historical context, perpetuates the controversy engendered by the Soviet authorities in 1945 about who invented radio. The question of Popov's contribution to radio was comprehensively and objectively examined by Susskind² in 1962. The conclusions were: (1) According to the criteria of priority of

publication, Marconi invented radio communication by making a successful application for the world's first patent for wireless in June 1896. (2) On the basis of historical research there is indirect evidence that Popov transmitted intelligent signals before that, in March 1896, but there is comparable evidence that Marconi did likewise at an even earlier date³.

Both Popov and Marconi, without knowledge of the other, developed radio receivers based upon the coherer design and decoherer invention of the Englishman Oliver Lodge⁴. Popov's first receiver (demonstrated in May 1895) was flawed. In Popov's words, its use for the transmission of signals over a distance might be achieved "as soon as a source of such oscillations with sufficient energy will be discovered"^{4,5}. Sufficient energy was found in powerful discharges of lightning and Popov's receiver was successfully used as a storm detector.

Meanwhile, Marconi had appreciated what Popov had failed to recognize — that intelligent communication required a sensitive receiver and not a powerful transmitter. Marconi had improved Lodge's coherer so much that during 1895 he transmitted Morse signals over a distance of 3 km and by February 1896 he was sufficiently confident with his invention that he left Italy for London to exploit a patent application⁶.

Marconi, in his own words, was "an ardent amateur of electricity" with an independence that allowed him to pursue his ideas. Popov was an eminent academic scientist, possibly constrained by being employed by the Russian Navy. As Susskind pointed out in 1962, the Russians have good reason to be proud of a pioneer of Popov's calibre, but to enlarge his reputation out of proportion to his achievements amounts to a deviation from objectivity that must be deplored².

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Polio vaccines

SIR — The eradication of poliomyelitis by early next century (*Nature* 374, 663; 1995) seems likely, but the end of the road is unlikely to be smooth and straightforward. This is obvious from a recent episode.

Two days before the start of a special

drive against poliomyelitis in India, 42 children were inoculated with polio vaccine at Nadia primary health centre (PHC) in West Bengal: within 24 hours, eight had died and 34 were seriously ill. This incident will have deterred hundreds of parents from vaccinating their children, thus wrecking their lives. Any immunization programme for successful eradication requires coverage of the target population. More than 75 per cent of new polio cases reported annually are from India, Pakistan, Bangladesh, Burma and Nepal. Because of poverty, a low rate of literacy and insanitary conditions, the wild virus thrives in this part of the world and poses a threat to regions free from polio. There were national polio immunization days all over the subcontinent in April. Middle East and Central Asian countries planned to immunize 70 million children, according to the World Health Organisation.

Many of the Indian daily newspapers on 5 April 1995 made the deaths at Nadia PHC their lead story. In fact what the PHC had used was not a vaccine at all. Even outdated polio vaccine is not lethal. Corruption in public health departments in some countries is another limiting factor in prompt and effective implementation of any massive programme. Large-scale theft and black market resale in Burma, and perhaps elsewhere in South-East Asia, of expired and potentially toxic vaccines for tetanus and diphtheria (see *Nature* 374, 669; 1995) is a glaring abuse. Criminal misuse and adverse publicity can cause parents to shun vaccination and jeopardize both the health of their children and the immunization programmes.

It is in everybody's interest that poliomyelitis should be eradicated as fast as possible. The money saved could be diverted to immunological studies. A better understanding of the immune system, which has evolved to combat pathogens, may help to deploy the future peptide vaccines efficiently against malaria and other eukaryotic parasitic diseases that have eluded control through vaccination by defined antigens. This type of investment, along with the European Commission plans for vaccine development (*Nature* 374, 663; 1995), should encourage pharmaceutical industries to manufacture newer and better vaccines that are expected to be developed more rapidly with advances in molecular biology.

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Letters submitted for Correspondence should be typed, double-spaced, on one side of the paper only.

Directory to the human genome

***Nature* plans to publish in the next few weeks a Genome Directory containing the latest catalogue of expressed sequence tags and refined versions of the physical map of the human genome.**

"If you publish this Venter stuff", said the distinctive voice on the telephone a few months ago, "I can promise you that nobody in the US genome community will ever send you anything ever again." The source of the voice, who will recognize himself, is one of the most distinguished geneticists in the United States. The "Venter stuff" is the compilation of "Expressed Sequence Tags" (or ESTs) corresponding to the ends of a large fraction of the genes in the whole human genome and constructed by J. Craig Venter, previously at the US National Institutes of Health (NIH) and now the operational head of The Institute of Genome Research (TIGR), a privately funded not-for-profit foundation under US law. Getting on for a year ago, the issue was controversial because Venter had offered to make his data generally available, but only on conditions that many people considered irksome.

What follows is an explanation of why *Nature* has nevertheless decided to publish "Venter's stuff", together with much else besides, despite the telephoned warning (no doubt uttered in the most friendly spirit). At some point next month, as a supplement to Volume 377 of *Nature*, Venter's latest compilation of ESTs will be published as part of what will be called the *Genome Directory*. That publication will also include the latest version of the physical map of the human genome from Génethon, the Paris-based not-for-profit organization whose best-known public representative is Dr Daniel Cohen. The *Genome Directory* will also include still more detailed physical maps of four human chromosomes as illustrations of how quickly the physical map of the human genome is being refined.

Availability

The *Genome Directory* will be sent to all institutional subscribers to *Nature* without any action on their part. In the belief (which may be misplaced) that many may not wish to be encumbered with such a mass of unsolicited data, personal subscribers are invited to complete the tear-out form facing this page. The directory will be supplied free of charge to registered subscribers. Unused copies may afterwards be offered for general sale.

This is probably the last occasion on which information of this kind will be available in print, as ink on paper. As will readily be seen when the *Genome Directory* appears, the information about the human genome now available can more conveniently

be obtained by computer-retrieval from one of the databases accessible through the Internet or otherwise. Among biologists, molecular geneticists are notorious users of computer time.

There are several good reasons (other than bravado) for publishing this material. The chief, as will be seen when the *Genome Directory* is distributed, is that the work described is excellent science with great intrinsic interest. From the outset, those planning the human genome project have acknowledged that their product would be no better than the techniques for making the data accessible and intelligible, but readers will be surprised at how much has been done to make the analysis of huge quantities of data manageable without the intervention of paper (which is the enemy of accuracy as well as speed).

Scale

The scale of these enterprises is also remarkable. Venter's group will be reporting that the total length of the ESTs now sequenced amounts to 5 million base-pairs, or some 0.15 per cent of the total length of the human genome. When overlapping and otherwise redundant sequences are removed from the catalogue, there are more than 55,000 ESTs that correspond to authentic genes, of which only some 10,000 are at present logged in public databases.

Cohen's activities are on a similarly huge scale. Fitting the markers onto the physical map has entailed that the whole genome should be cloned by incorporating pieces of it into yeast artificial chromosomes (YACs). The solution of the resulting jigsaw puzzle — which YAC comes next? — when there are several thousands of pieces, only parts of which are known by their nucleotide sequence, is a task that can be solved only by a computer and the appropriate software.

There are good reasons why data of this kind should be generally available, even if for the last time in the traditional manner. One is that the data will vividly illustrate that the next step, for those wishing to describe the organization of the human genome, is to relate Venter's (and other people's) ESTs with Cohen's physical map of the genome.

Cohen's map would not have been possible without the marker set developed by Jean Weissenbach, which is the outcome of a decade-long research programme begun at the Centre d'Etudes Polymorphisme Humaine (CEPH) in Paris. The construction of a physical map has been done under

the umbrella of Génethon (a charity concerned with muscular dystrophy which raises some of its funds through telethons). The markers are short DNA sequences defined as such, meaning that they can be transferred to other laboratories on paper or by e-mail. They are, as far as it is possible to tell, unique within the human genome.

Given the original goal of the CEPH programme, which is to use markers as a way of locating genes associated with inherited disease, it is natural that the bulk of the markers should be polymorphic in the sense that the sequences may differ between one individual and another. When the precise location of a gene is not known, the inheritance of a particular allele at a polymorphic site is a good proxy for the inheritance of the gene or, conversely, the location of a disease-linked gene can be estimated approximately by telling which of several polymorphic markers has a pattern of inheritance most closely correlated (or 'linked') with the pattern of clinical disease. The utility of Cohen's markers rests on painstaking pedigree analysis by CEPH as well as on the careful physical location of the markers on particular chromosomes that he and his colleagues describe in their forthcoming paper.

ESTs

ESTs, by contrast, are generally free from polymorphism. Made as they are by converting cellular messenger-RNA (mRNA) molecules into complementary or cDNA, they should correspond to the coding portion of the gene whose transcription formed the mRNA concerned. That means that ESTs as such can be of little value in the preliminary investigation of genetic diseases caused by mutations in a gene (or genes) unknown, where linkage studies are the classical starting-points. The real benefit of a combined map annotated with ESTs would be that known genes recognizable by ESTs could be pulled out from the neighbourhood of any suspect linkage marker by reference only to their ESTs. The ten-year delay between the linkage studies showing the approximate location (within a million base-pairs) of the gene implicated in Huntington's disease and its eventual identification is a measure of the benefits that will accrue.

But that is only the first step. When the ESTs have been placed on a physical map, speculations about the ways in which groups of genes are regulated in concert will become much more tangible. The *Genome Directory* will show that Venter and his asso-

ciates have been searching for ESTs in cDNA derived from more than 30 different tissues in the human body. That is evidently a potentially fruitful approach to the question of how differentiated cells differ from each other genetically. The comparison of EST catalogues from different tissues and from different species will evidently be an important tool in basic research in biology for many years to come.

Of course, the more immediate value of access to a catalogue of ESTs is that it will simplify the search for genes implicated in disease as well as the identification of the function of those same genes in people free from disease. That is why the pharmaceutical companies are intensely interested in these developments; they offer a route to the devising of medicines that may prevent the production of an unwanted gene product or make up for the loss of biochemical function as a consequence of a mutation.

So why should the offer to make these data available to the academic research community, but on restrictive terms, have caused such a furore last year? (The controversy attaches only to the EST catalogue.) TIGR is funded privately, but has an agreement with the frankly commercial organization called Human Genome Sciences (HGS), which is itself a major source of the cDNA libraries and of the analytical work with which the new EST catalogue has been constructed. HGS has the first right to exploit commercially the growing EST database that it is developing with TIGR, but HGS also has an agreement with Smith-Kline Beecham, the pharmaceutical company, which gives that company first access to its own rights.

Access

At the outset, a year ago, TIGR and HGS announced that their EST catalogue would not be made available through the public databases (GenBank, for example), but that academic members of the research community could apply for the right to search their server for data after first signing one of two access agreements. It was to be a part of the agreement that physical samples of the ESTs in the catalogue would also be provided. But those requesting access were required to undertake that discoveries leading to potentially valuable commercial applications would first be disclosed to HGS, which would have the right to exploit them. Other strictly commercial organizations would be denied access.

Inevitably, there were several objections to these policies, ranging from the argument of the Human Genome Organisation (HUGO) that such an array of such basic information about the structure of the human genome should be in the public domain as a matter of principle to a proposal that there should be an annual limit to the number of ESTs that a properly signed-up investigator could retrieve. Certainly the second restriction would have prevented the

use of the catalogue of ESTs by those eager to compare the occurrence of large numbers of genes in different human tissues, for example.

The position of TIGR and HGS, last year and this, is that their EST catalogue is legitimately proprietary information which has been gathered with private, not public, funds, and to which restrictions of investigators' freedom to carry through commercial applications are common and commonly accepted, even in dealings between largely academic laboratories. The question of whether ESTs (and DNA sequences generally) are patentable is, in this connection, a red herring. When Venter listed his first 600-odd ESTs four years ago, NIH applied for patents and was eventually refused, but the issue remains open, especially in the light of a recent federal court decision in the United States that a particular gene sequence should be given patent protection. But the reality of the present situation is that SmithKline, HGS and TIGR have access to a database they can use as a tool for commercial purposes, and to which others have only restricted access.

Response

One significant response to this situation is that of the US pharmaceutical company Merck, which has provided a grant of \$10 million to Washington University at St Louis, Missouri, to replicate the construction of an EST catalogue entirely in the public domain. DNA sequences from this project are being added to GenBank at the rate of several hundreds every week. That is an excellent development, although it will not immediately make it possible for institutions with an urgent interest in the TIGR/HGS database to avoid negotiating an access agreement. In the long run, much will depend on the reliability of the information from the two sources.

All the articles in the *Genome Directory* have been refereed in the usual way. The proposed terms on which access to the EST catalogue will be granted have been an important ingredient of that process. Each of the two geneticists who undertook the task had specific criticisms to make of the arrangements proposed by TIGR and HGS. In the opinion of the two referees and of *Nature's* editors, the specific objections have been met by the provision of extra computer equipment, user manuals and the like. Except for the undertaking on commercial exploitation, access is virtually unrestricted. (A third referee, asked to check the integrity of the database, reported that it functions as described, but offered his objection to the TIGR/HGS initiative. People wishing to search the whole database, for example, may have to do so at night, when other demands do not conflict.)

In *Nature's* judgement, the publication of this material in the *Genome Directory* is appropriate, given the interest of the field and the circumstance that the restrictions

now placed on access to the data are no more onerous than they would be for some other repository of proprietary information. Indeed, in the real world, such information is usually emphatically unavailable, as geologists eager to search through petroleum exploration companies' drilling logs will readily confirm. It would be a loss to the research community, and a disservice to the authors of this material, if there were to be no record of it at this critical stage in the evolution of the human genome project.

Exactly what will happen to the human genome projects in the years ahead is now less clear than it has been. If the objective were simply the construction of a catalogue of all the 100,000 or so human genes, the ease with which ESTs can be derived from genome libraries has evidently brought the goal much closer than seemed possible five years ago. Many in the genome community now fear that the US Congress, particularly in its present penny-pinching mood, will regard the emergence of two catalogues of ESTs, both privately financed, as a sufficient substitute for the sequencing of the entire genome at an estimated cost of \$3 billion spread over a decade.

That response, of course, is unwarranted — and the fear it provokes is probably misplaced. Largely at the urging of the members of HUGO, often in their personal capacities, there has emerged an international pattern of research with a reasonable division of labour and a substantial momentum of its own. For example, the genome of the nematode worm *C. elegans* is being carried through jointly by Washington University and the Sanger Centre at Cambridge, UK. *Arabidopsis* chromosomes have been neatly parcelled out between European laboratories. At least three of the yeast chromosomes have been fully sequenced; the others will trickle out in the next 18 months.

Apart from the intrinsic interest of that information, these preliminary projects will be what they were intended as; ways of anticipating technical difficulties in comprehensive sequencing and pointers to the arrangements there will have to be for handling the much greater volume of data spawned by the human genome project proper. It will soon be time to effect a division of labour on that bigger task. There is little doubt that the methods developed by Cohen as well as Venter will inspire much of the planning that lies ahead.

But why bother with the whole genome, when ESTs will allow the identification of all the genes? That is what the Congress will be asking. The answer is that experience so far shows clearly that the locations of all the genes will not, of itself, indicate how the genome functions. Nor will it throw much light on the question of how the genome became what it is in the course of human evolution. Answers to those questions are no less important and interesting than the uses to which ESTs will be put in the immediate future.

John Maddox

American ecology at the crossroads

Faced with a hostile Congress, US ecologists must distance themselves from environmental activism, and perhaps adopt lessons learned in Britain.

Snowbird, Utah. "When many in our Congress attempt to overturn carefully crafted laws and regulations that protect our citizens from pollution and the looting of our natural heritage, they are ... attempting to overturn a 500-year legacy of civilization that has steadily replaced superstition and irrationality with understanding of, and respect for, science".

It is language like this from the President's Men — in this case Mr Timothy Wirth, Under Secretary of State for Global Affairs — that has got Republican congressmen rattled (see page 453). Wirth could pull it off, as he was addressing the home crowd, at the 80th annual meeting of the Ecological Society of America in Snowbird on Tuesday last week (1 August). His words were balm to researchers bloodied by a Congress generally hostile towards their work. Ecologists are seen as enemies of the free market, whose work inevitably leads to oppressive regulations to protect endangered species and bar economic progress. The reaction of politicians is twofold: either they foster a culture of ignorance (in Wirth's words "what we don't know can't hurt us — or take responsibility for"), or they tar ecologists with the brush of environmental activism, emphasizing connotations of left-wing ecoterrorism, abrogation of the rights of private landholders, and frankly un-Christian moral laxity.

One victim has been the Biodiversity Treaty discussed at the United Nations conference at Rio de Janeiro two years ago. After much scholarly and political discussion, and having been passed by the Senate Foreign Relations Committee by 16 votes to 3, it was derailed when a right-wing commentator produced a 75-page report evoking, in Wirth's words, "images of world governance, paganism, nature worship and a host of other nonsense". Ratification of the treaty by the United States seems to have been postponed indefinitely.

Salem is dangerously close, but if working ecologists are in the habit of lewd exhibition in the woods by night, ululating before graven images of Pan, they are surely joined by those notorious left-wing intellectuals former Presidents Ronald Reagan and George Bush: for another move seeks to reverse the Montreal Protocol on the emission of chlorofluorocarbons (CFCs), an agreement based on programmes of research into climate change encouraged by those two regulato-

ry zealots. On the basis of reports by individuals who, according to Wirth, lack the appropriate expertise, Arizona has passed a law allowing the manufacture of CFCs.

Some elements in Congress are clearly having a field day. But if, to paraphrase Emerson, against stupidity the gods themselves contend in vain, how can ecologists here hope to stave off the oncoming fall of night?

The problem is more of public image than the research itself. Ecologists should distance themselves from unfocused environmental activism, promoting the kind of serious research that will make a positive difference to the health and wealth of the United States. People get the governments they deserve, but in the last congressional poll, few voted for polluted drinking water or the barbecuing of the last spotted owl. To this end, ecologists could learn from the British experience. In respect of governments with all eyes on the bottom line, the United Kingdom has 16 years' headstart on the current Congress (although to be fair, the British government, for all its myopia, has always been far less hostile).

Before Rio, the public perception of ecology in Britain was of synonymy with activism. At that time it was routine to see, at serious meetings, hirsute, mad-eyed men in chunky knitwear exhorting us to save the lesser swivel-eyed bandicoot (just substitute your favourite cuddly species here), and saying that all endangered species must be protected, no matter what. Although, even then, arguably only a minority struck such attitudes, their clear message attracted the most attention. The establishment response was one of indulgent sympathy, but ecologists got the message that these Rousseauesque fantasies, although noble, provided little guidance for the formulation of policy.

In response, organizations such as Greenpeace replaced rhetoric with research. Environmental protection became a respectable, mainstream concern: even the then Prime Minister, Mrs Margaret (now Baroness) Thatcher, was at pains to demonstrate her 'green' credentials.

Ecologists began to ask questions based on the assumption that, given realistic and finite budgets, hard choices had to be made. To inform such choices, they devised operational definitions for such hitherto nebulous terms as 'biodiversity', which would be useful in practical deci-

sion-making. The league of hirsute, mad-eyed men was dislodged by a new breed of ecologists in coat and tie, no stranger either to differential equations or to the corridors of power. It is perhaps no accident that Professor Robert May of the University of Oxford, an ecologist of this stripe, is now the chief scientific adviser to the British government.

None of this is meant to imply that ecology is less rigorous in the United States than elsewhere. Rather, the poor public image of US ecologists among politicians, already inclined to hostility, makes changing their minds more difficult than it might otherwise be.

The first task is to counter, calmly but firmly, the continuing political campaign of misinformation. This already takes up much of the time of Dr Ron Pulliam, director of the National Biological Service (NBS). Formed less than two years ago from the research arms of the Fish and Wildlife Service and other bodies, the NBS is as endangered as the spotted owl and may yet go the way of Florida's dusky seaside sparrow (deceased 16 June 1987).

To the cynic, the formation of the NBS from bodies with more diverse functions looks like something set up only to be knocked down again. If the recipe for congressional cuts is a Shakespearean witches' gruel, thick and slab, the NBS falls too easily into the role of birth-strangled babe, ditch-delivered (in Republican eyes) by a drab.

The second task is to promote the kind of level-headed compromise not usually associated with activism. This attitude might be successfully applied to the Endangered Species Act, due for reappraisal in Congress. The likely damage, though, will surely be limited if ecologists can draw the congressional sting by making it plain that there are compromises they would be prepared to accommodate. Many ecologists clearly feel this way, but their message is not getting through.

Not that ecologists could not recruit a little outside help, perhaps from unlikely places. Members of the National Rifle Association, whose liberal views are so cleverly masked by their vocal assertion of their constitutional right to bear arms, should extend this to bearing them into uncut forests for the purposes of bagging abundant wildlife.

As every ecologist knows, selective pressure and habitat disturbance creates surprising bedfellows.

Henry Gee

Keeping enhancers under control

Victor G. Corces

THE increase in genome size that accompanied the evolution of higher eukaryotes poses the serious problem of how to present the huge amount of regulatory information in DNA to transcription factors while keeping tight control of gene expression during the development of a particular organism. For example, how do genes prevent their own enhancers from activating transcription of neighbouring genes? Chromatin insulators seem to be in charge of this particular job, and a paper by Cai and Levine¹ on page 533 of this issue goes a long way towards explaining how they might accomplish it.

It has been proposed that some of the

regions (LCRs) which define functional domains of gene expression³. LCRs are clusters of regulatory sites that are needed for appropriate and regulated levels of expression of a group of genes. It now seems that LCRs contain a third type of domain indicator, a boundary or insulator, which is defined as a sequence that prevents enhancers located on one side of the boundary from acting on promoters located in the adjacent domain⁴.

The *scs* and *su(Hw)* binding sequences discussed by Cai and Levine¹ are examples of such boundaries. The stripe 2 and stripe 3 *eve* enhancers are normally located within the same chromatin domain

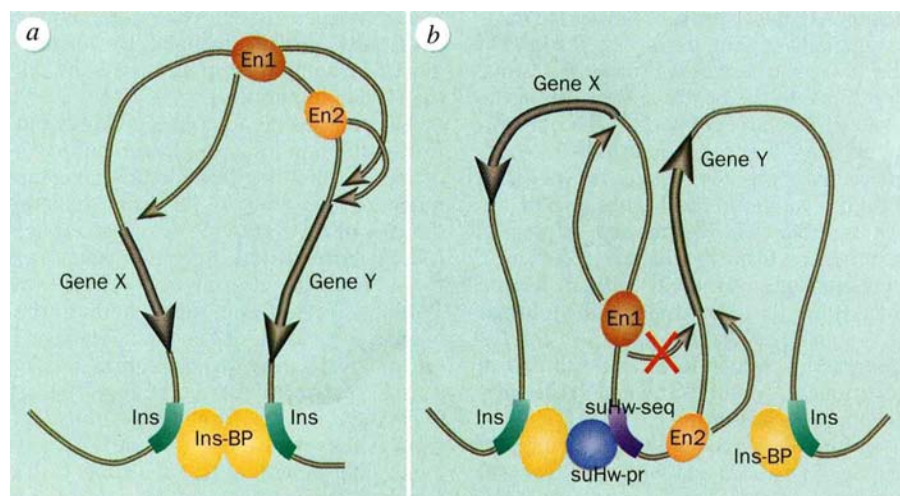
as the *eve* promoter; this domain is flanked and defined by two boundary elements (*a* in the figure). If an insulator such as the *su(Hw)* or *scs* sequence is interposed between one of the *eve* enhancers and the promoter, this enhancer becomes segregated into a different chromatin domain and is now unable to communicate with the transcription complex located in the *eve* promoter (*b* in the figure).

Recent progress in the characterization of proteins that bind to boundary elements and are responsible for their insulating properties gives fresh insight into the mechanism of action of these elements. Laemmli and colleagues⁵ have identified a protein named BEAF-32 that binds to the *scs*' boundary element and is responsible for its insulating properties. Interestingly, BEAF-32 is localized on interbands in *Drosophila* polytene chromosomes, suggesting that chromosomal domains defined by the *scs*' element consist of a band plus part of the flanking interbands. The *scs*' sequences fail to provide insulating properties in transiently transfected cells in tissue culture, suggesting the involvement of chromatin or nuclear localization in their function.

Similarly, two proteins encoded by the *su(Hw)* and *mod(mdg4)* genes have been found to be necessary for proper function of the *su(Hw)* insulator⁶, and both proteins seem to be capable of affecting chromatin structure. Binding of the *su(Hw)* protein to its target sequence creates a bidirectional repressive effect that is similar to the silencing caused by heterochromatin; subsequent interactions between the *mod(mdg4)* and *su(Hw)* proteins transform this nonspecific silencer into a polar insulator.

The mechanisms by which insulators affect enhancer function in this polar manner will necessarily depend on how enhancers affect transcription activation from specific promoters. Simple models viewing insulators as roadblocks that interfere with tracking, or as sinks that sequester enhancer-bound transcription factors, are easy to visualize but fail to account for the observed correlation between insulator function and changes in chromatin structure. It has been proposed that enhancers act by causing the spreading of an open chromatin conformation from the enhancer to the promoter⁷, and many properties of insulators could be explained by an effect on enhancer-induced chromatin remodelling. These effects on chromatin must be compatible with the finding by Cai and Levine that an insulated enhancer is still able to function on a promoter located within the same chromatin domain, and with the finding indicating that RNA polymerase is able to transcribe through an insulator⁸.

Many questions still remain to be answered before we understand the structure and role of chromatin insulators, but their unique properties lend themselves



Organization of a chromatin domain. *a*, Insulator sequences (Ins) define the limits of a chromatin domain isolating the genes contained within their boundaries from the transcriptional influence of sequences outside the domain. The insulating effect is mediated by proteins (Ins-BP) that bind to these sequences. Enhancers (En1, En2) that control the expression of gene Y are also able to influence the expression of gene X located within the same domain. *b*, When an insulator such as the *su(Hw)*-binding region (*suHw-seq*), which interacts with the *su(Hw)* protein (*suHw-pr*), is interposed between the two gene Y enhancers, a rearrangement of chromatin domains takes place such that En1 is in one domain whereas gene Y is in a different one. En1 is now unable to act on the gene Y promoter, but can still activate transcription of gene X.

organizational properties of the eukaryotic genome reside in the ability of chromatin to organize autonomous functional units that delimit levels and patterns of gene expression. The existence of these domains was originally inferred from cytological studies on insect polytene chromosomes and the lampbrush chromosomes of amphibian oocytes. Since then, these domains have been defined further on the basis of structural and functional analyses.

Structural studies of chromosome organization led to the discovery of SARs or MARs, which are DNA sequences that mediate the attachment of chromosomes to the chromosome scaffold or nuclear matrix and structurally delimit chromatin loops². Furthermore, functional analysis of gene expression in transgenic mice resulted in the finding of locus control

as the *eve* promoter; this domain is flanked and defined by two boundary elements (*a* in the figure). If an insulator such as the *su(Hw)* or *scs* sequence is interposed between one of the *eve* enhancers and the promoter, this enhancer becomes segregated into a different chromatin domain and is now unable to communicate with the transcription complex located in the *eve* promoter (*b* in the figure).

The critical question now is to define the mechanisms by which boundaries or insulators repress enhancer function in a polar manner: that is, only enhancers on one side of the boundary are affected, whereas those located between the boundary and the promoter are active. An important step in solving this puzzle is the discovery by Cai and Levine that enhan-

to endless possibilities for controlling eukaryotic gene expression. Although there is no homology between boundary sequences identified so far, hybrid chromatin domains might be formed by the interaction between two different insulators if the proteins that constitute these entities are promiscuous and able to interact with each other. The presence of tissue-specific boundary proteins would then allow flexibility in establishing higher orders of chromatin organization, enabling fluid chromatin domains to form that could change in response to developmental programmes. Insulators might also come in different flavours and strengths. Different affinities among the protein components of the insulators could give rise to a hierarchy of chromatin domains with varying abilities to affect enhancer-promoter interactions, depending on the

strength of these two transcription signals. The possibilities are unlimited and only time will tell whether insulators are able to impose some order on the puzzle of eukaryotic gene regulation. □

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CLIMATE CHANGE

A successful prediction?

Tom M. L. Wigley

THE ultimate test of the current crop of coupled ocean-atmosphere general circulation climate models is their ability to simulate past climate variations. In the most sophisticated model experiments yet performed, Mitchell and co-workers¹ claim, guardedly, to have done this. Their work is described on page 501 of this issue. Because the climate system is so complex, the task is an exceedingly difficult one, so one must view the results circumspectly. Nevertheless, when considered along with other recent work, these results mark a turning point in our ability both to understand past changes and predict the future.

Why is this such a difficult problem? Climate models are basically extended versions of models used for weather forecasting, which have a high success rate. The difficulties arise in the extension. Because climate studies concern time-scales much longer than weather forecasting — from years to decades — the slowly varying ocean, which acts as the flywheel of the climate system, must be considered. Thus, atmospheric models that are used to study weather must be coupled to equally complex models of the ocean if we are to analyse the climate. This introduces a new problem, that of fitting together the atmospheric and oceanic jigsaw pieces, a problem that is yet to be adequately solved. In modelling jargon this is referred to as the 'flux adjustment' problem². In addition, sea ice variations and their interactions with the ocean and atmosphere must be included, introducing further scope for model error and misfits between the components. Finally, the importance of correctly modelling cloud processes

becomes amplified because these are a crucial factor in determining the model's 'climate sensitivity' — how much warming will eventually occur for a unit change in external radiative forcing³. Weather systems do not care a jot about the climate sensitivity.

To increase the difficulty, even if we had perfect models, we would not be able to simulate the past (or the future) unless we knew the history of the natural and human-induced factors that have conspired to change the climate, collectively called 'external forcing'. Until recently, all climate simulations have supposed that the only anthropogenic forcing was carbon dioxide (sometimes scaled up to account for other greenhouse gases). We now believe that other factors may be of similar importance to greenhouse gases, particularly sulphate aerosols. These are small particles that derive, like carbon dioxide, from fossil-fuel combustion. Their effects, however, oppose the greenhouse warming effect, at least regionally. Under clear sky conditions, they reflect incoming solar radiation back into space (a direct effect), while in clouds, by changing the size distribution of water droplets, they make those clouds more reflective (an indirect effect)⁴. Both processes cause cooling, but the pattern of this cooling is vastly different from the pattern of greenhouse warming, so the combined climate influence is complex.

The study by Mitchell *et al.* is the first to be published in which both carbon dioxide and aerosol influences have been examined with an ocean-atmosphere general circulation model. The coupling of ocean and atmosphere marks an impor-

tant advance over work last year by Taylor and Penner⁵, who used a model with a simpler ocean that allows only equilibrium results (rather than time-varying results) to be obtained.

There are other important differences between these studies. Taylor and Penner modelled the full aerosol system, although they considered only the direct radiative effect. Mitchell *et al.* do not explicitly include aerosols at all; rather, they simulate their effect by changing the model's surface albedo or reflectivity. Although their focus is on the direct effect, they may fortuitously cover the indirect effect because both have similar patterns of forcing^{6,7}. A crucial factor in their work is the magnitude that they assume for the global-mean aerosol forcing, about 0.6 W m^{-2} in 1990. Although this is large relative to what we currently believe for the direct forcing, it is small relative to best-guess values for the total forcing. The word 'guess' is not used idly here — the uncertainties surrounding the magnitude of aerosol forcing in general and sulphate aerosol forcing in particular are exceedingly large. These uncertainties are central to any assessment of Mitchell and co-workers' results.

Their simulation of global-mean temperature changes from 1861 to present shows good agreement with observations, far better agreement when aerosols are included than when they are not. This, in itself, is not a new result; indeed S. Raper and I, in work that considered both climate sensitivity and aerosol forcing uncertainties⁸, have shown that aerosols improve the fit with the past provided that the aerosol forcing is relatively low — as it is in the work of Mitchell *et al.* However, we were using a much simpler model, so the new result adds considerable credibility to the conclusions.

Mitchell *et al.* also examine the time-varying patterns of temperature change. Here they find increasing similarity with the observations over the simulation period, just as one would expect to happen as the 'signal' of anthropogenic climate change rises above the 'noise' of both the model's and the real world's natural variability. This increased pattern correspondence confirms work by Santer and co-workers⁹, who searched the observed record to see how well it corresponded to the pattern of the equilibrium temperature change in Taylor and Penner's⁴ results. They also found an increasing pattern similarity with time. One must be cautious not to over-interpret these results, because they are based on models with clear deficiencies. However, global warming aside, it is surely more than mere coincidence that, when we improve the realism of the searched-for anthropogenic signal (by including aerosol effects) we obtain the positive results that eluded us before. ▶

Where next? Mitchell *et al.* have already taken the first step by running their model through to 2050. For the first time, we have results for the future that, because they include aerosols, are based on a realistic foundation. They predict that, in the period from the pre-industrial age to 2030–50, warming still occurs almost everywhere, although the warming is less marked when aerosols are included than when they are not. The effects of aerosols are most evident in North America and across central Asia to China. Unfortunately, the changes that would be more relevant to policy decisions between now and 2030–50 are not shown — we must still wonder what the effects of the projected east–west shift in sulphur dioxide emissions will be.

Clearly, there is much yet to be done. Mitchell *et al.* only consider one picture of future emissions: there is a whole range of possibilities depending on projections of population and economic growth. To span the range of likely futures, many other scenarios must be examined. Furthermore, although the results show encouraging agreement with observations, there are still enormous uncertainties related to the overall sensitivity of the climate system and the strength of the

aerosol forcing effects. It could be that Mitchell and co-workers' agreements are the results of compensating errors, too low a sensitivity coupled with too low an aerosol forcing effect. The consequences of such errors for the future would be large.

So far, climate modellers have had limited successes and have had to bear the brunt of criticism from those who are concerned about the role of models in the greenhouse policy debate. At last, however, it seems that the door is opening and the light of credibility is filtering through. □

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ANTIGEN PRESENTATION

DM exchange mechanism

Paula R. Wolf and Hidde L. Ploegh

T LYMPHOCYTES recognize their targets by focusing their attention on small peptides displayed at the cell surface in a complex with the products of the major histocompatibility complex (MHC). There are two types of MHC molecule, class I and class II, with homologous functions, but distinct in the intracellular site at which they pick up their peptide cargo. Peptide loading of MHC class II molecules is a complicated, multistep process, and new components in this processing and presentation pathway continue to be discovered. We now learn of the function of one such recent addition, the DM molecule, in papers appearing in *Nature*¹, *Cell*² and *Immunity*³.

MHC class II molecules are $\alpha\beta$ heterodimers that scour the endocytic pathway for antigenic peptides (see figure). They reach this destination by association early during biosynthesis with a membrane protein, the invariant chain (Ii), whose proteolytic destruction is a prerequisite for peptide loading onto the $\alpha\beta$ dimers. Surprisingly, the presence of MHC class II products alone is not sufficient to ensure antigen presentation. Analysis of mutant cell lines has revealed additional genes whose products are required to orchestrate a class II-restricted T-cell response:

HLA-DMA and *HLA-DMB*, which encode proteins that are class II-like in nature but whose function is unknown^{4–6}.

During the course of destruction of Ii, peptide remnants that include residues 81–104 of Ii (designated CLIP, for class II-associated invariant-chain peptides) remain tightly associated with class II in the peptide-binding groove (P. Gosh, M. Amaya, E. Mellins and D. Wiley, manuscript submitted). Exposure to low pH and proteases is not sufficient to remove CLIP, and it is here that DM is required. In the absence of DM, class II molecules are not loaded with antigenic peptide, but are expressed instead on the cell surface in a complex with CLIP peptides^{7,8}. The three new studies provide direct evidence that the DM products, at least *in vitro*, serve to liberate the class II antigen-binding site and facilitate loading with antigenic peptides^{1–3}.

In the antigen-presenting cell, binding of peptide to class II molecules occurs very rapidly, at a rate that cannot be matched *in vitro* by purified class II molecules. The missing catalyst is DM: reconstitution of purified mature class II molecules into DM-containing membranes allows rapid peptide loading *in vitro*³. The simple exposure of class II $\alpha\beta$ -CLIP complexes to

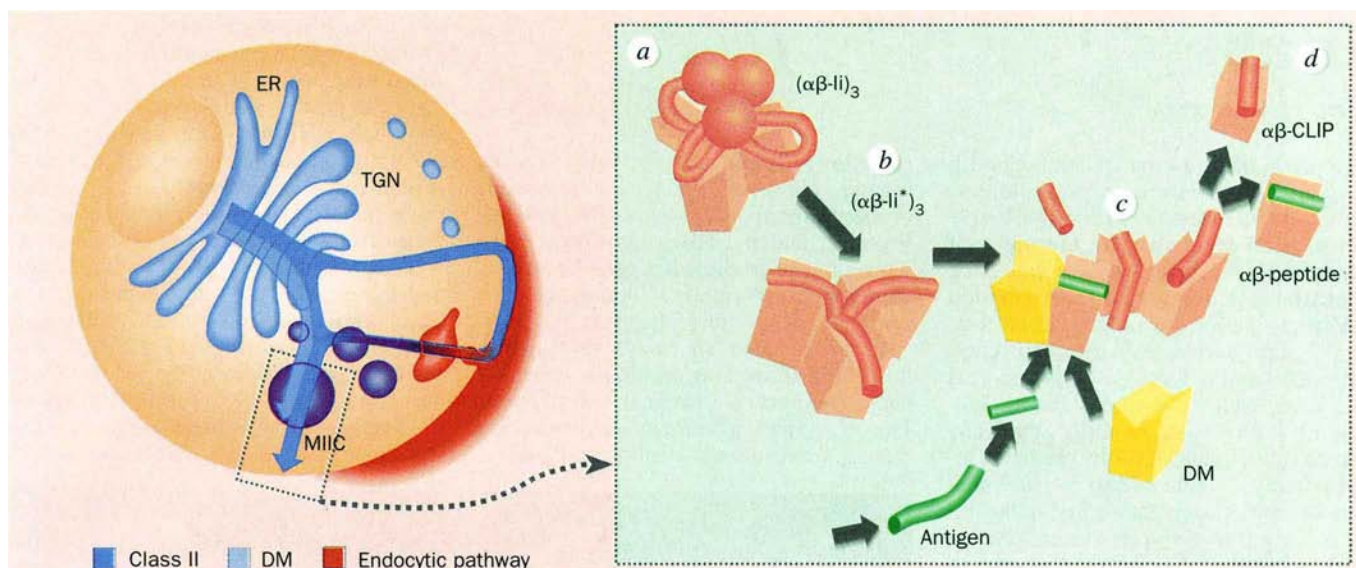
DM at low pH *in vitro* releases CLIP and increases the binding of peptide by class II^{1–3}. Moreover, addition of recombinant soluble DM enhanced the amount of peptide bound by both peptide-occupied and 'empty' DR molecules in a plate-binding assay¹. The affinity of DR for peptide was not altered by soluble DM: rather, it made the antigen-binding site of DR more available to peptide. Accordingly, the presence of soluble DM increased the dissociation rate of CLIP peptides bound to DR.

Equivalent results were obtained using DM and DR molecules isolated from cell lysates^{2,3}. At low pH, immunoprecipitated DM induced the release of CLIP from $\alpha\beta$ -CLIP complexes³ and, in the presence of appropriate peptide, rendered these complexes stable in the anionic detergent SDS, a feature of mature, peptide-loaded class II molecules².

The kinetics of SDS-stable dimer formation and CLIP removal imply that these two steps are coupled and support a catalytic function for DM. To explain this increase in 'availability' of the peptide-binding groove, the fleeting interaction of DM with a class II-CLIP intermediate must be accompanied by subtle conformational transitions in the class II molecules themselves. Roles for DM as a sink for Ii breakdown products or as a chaperone to direct class II molecules to the site of action no longer sound so attractive.

The DM α - and β -subunits are structurally similar to class II α - and β -chains. Completely unrelated functions for MHC products and their close relatives are not without precedent. For example, although the primary structure of the neonatal Fc receptor (FcRn) and the identity of its light chain, β_2 -microglobulin, loudly advertise its class I nature, it took the three-dimensional structure to reveal a warped peptide-binding pocket that has lost the ability to engage peptides⁹. Notwithstanding the remarkable structural conservation, both the manner in which FcRn binds its ligand and its biological function are completely distinct from those of its close cousins, the class I molecules.

Class II α - and β -chains form a heterodimer with a peptide-binding cleft, yet there are no data to suggest that DM can in fact bind peptide. Unlike conventional class II molecules, DM does not appear to associate with Ii, and is absent from the cell surface. The DM $\alpha\beta$ dimer reaches its final destination in the endocytic pathway independently of Ii, by means of its own targeting signal located in the cytoplasmic tail of the DM β -subunit (M. Marks, personal communication). DM, class II-Ii complexes and processed antigen all meet in structures having late endosomal/lysosomal characteristics and designated as MHC class II compartments (or MIIC)^{4,10–13}. How peptide-loaded class II



Left, the key to MHC class II-restricted antigen presentation is at the intersection between the biosynthetic pathway of MHC class II molecules (light blue arrow) and the routes travelled by endocytosed proteins (red). MHC class II molecules travel from the endoplasmic reticulum (ER) via the Golgi to endocytic structures referred to as MHC class II compartments, or MIIC. They may reach this destination either directly from the *trans*-Golgi network (TGN) or after insertion at, and retrieval from, the cell surface (dark blue arrow). The relative importance of these pathways is still controversial, but there is agreement that sorting signals borne by the cytoplasmic tail of the invariant chain (Ii) are of overriding importance in the delivery of class II molecules to MIIC. DM molecules reach MIIC of their own accord (light blue arrow), independently of Ii but aided by signal(s) borne by the cytoplasmic tail of the DM β -subunit. Proteases in the endocytic pathway destroy antigenic proteins and generate peptides for presentation by class II molecules. It is assumed that 'trimming' of peptides is complete before interaction with class II molecules. These same proteases destroy Ii and free up the peptide-binding pocket. The expanded picture of MIIC

(right) shows class II $\alpha\beta$ heterodimers (pink) assembled onto a trimer of Ii (dark pink) to form a nonameric structure $(\alpha\beta-Ii)_3$ (a), of which only the luminal portion is represented. This structure is attacked by proteases in the endocytic pathway, where the luminal domains of Ii are likely to be destroyed first (b), leaving a membrane-attached Ii remnant. This fragment probably associates with class II molecules through the so-called CLIP segment (residues 81–104) of Ii. DM (yellow) is proposed to interact with class II–peptide complexes, in which the peptide may either be the Ii-derived CLIP segment or a larger CLIP-containing fragment of Ii (dark pink), or — less likely — an antigenic peptide already bound (green) (c). Peptides that interact with class II molecules sufficiently strongly to survive this encounter will persist and be exported to the cell surface (d). Class II–CLIP complexes that fail to interact with DM because DM is in short supply will remain as class II–CLIP complexes, which may also be displayed at the cell surface (d). The true target of DM could be the nonameric complex of $\alpha\beta-Ii$ remnants (b and c), the dissociation of which would be promoted by interaction with DM.

molecules escape from MIIC to the surface is not yet known.

Given the long half-life and apparently sub-stoichiometric amounts of DM *in vivo*, each DM molecule is likely to be used repeatedly to generate class II–peptide complexes. However, it took a 10-fold excess of recombinant soluble DM over DR to induce both CLIP release and peptide binding to class II molecules¹. It was reassuring that the amount of immunoprecipitated DM required to convert $\alpha\beta$ -CLIP into $\alpha\beta$ -peptide complexes was comparable to the levels available *in vivo*, with $\alpha\beta$ -CLIP in excess over DM². These discrepancies probably result from the different assay conditions.

Does this mean that we now know how DM performs its job? Immunologists are only too familiar with the limitations of the *in vitro* approach: throwing synthetic peptides at cells is an effective but crude imitation of the cell's own sophisticated machinery for generating peptide–MHC complexes. Highly informative it may be on the specificity of the interaction between the T-cell receptor and MHC–peptide complexes, but few would consider this mode of peptide loading as physiological. The new results for DM^{1–3} are encouraging, but the identity of its

substrate will need to be verified in a more physiological setting.

Upon delivery to MHC class II compartments, DM may encounter $\alpha\beta$ -Ii nonamers and their breakdown intermediates before seeing the $\alpha\beta$ -CLIP complex (see figure). Denzin and Cresswell demonstrate that DM is unable to convert $\alpha\beta$ -Ii nonamers into peptide-loaded molecules *in vitro*, but is capable of using a breakdown intermediate². Some luminal trimming of Ii may thus be required to render class II molecules accessible to DM. Because partially degraded nonamers are not stable in solution, the dissociation of $\alpha\beta$ -Ii nonamers into individual $\alpha\beta$ -Ii trimers is perhaps all that is required for DM to exert its effects in the environment of the MIIC. The data indicate that a transient interaction must

occur: an anti-CLIP antibody blocks DM-mediated peptide loading of $\alpha\beta$ -CLIP complexes², and DM is incapable of exerting its effects on a mutant DR molecule containing an additional *N*-linked glycan at position 94 of its $\alpha 2$ domain¹. This glycan is predicted to point away from the interface that stabilizes the class II 'dimer of dimers'¹⁴, implying that the product of the DM–class II interaction is different.

The next step, if not the end of this story, should surely be the determination of the structure of $\alpha\beta$ -Ii complexes at atomic resolution. □

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An absorbing mystery

Warren J. Wiscombe

In January, after a year of duelling with angry critics in conference presentations and seminars, Cess *et al.*¹ published a controversial challenge to the field of atmospheric shortwave radiation. Using collocated satellite and surface radiation data from five locales ranging in latitude from Point Barrow, Alaska, to Cape Grim, Australia, Cess *et al.* found that cloudy atmospheric columns absorb substantially more solar radiation than any current model can possibly predict². Independently, Ramanathan *et al.*³ and Pilewski and Valero⁴ published measurements from the tropical western Pacific which agreed with Cess's group's findings. If these findings are true, the additional amount of solar energy absorbed in the atmosphere, and thereby denied to the Earth's surface, is large enough to alter dramatically our notions of how the current climate works. It would cause the biggest change in our understanding of the Earth's energy balance since the late 1960s, when satellites showed that the Earth reflects only 30 per cent of the incident sunlight, not 35–40 per cent as previously thought.

On page 486 of this issue, Zhanqing Li *et al.*⁵, using a different and geographically more extensive surface radiation data set than Cess *et al.* used, find that the increased absorption occurs only in warm seasons — mainly in the tropics and mid-latitude summers. At other times, they find a tendency towards greater absorption than predicted by current models, but far less than in the results of Cess *et al.* They furthermore find that the absorption effect is highly variable at all latitudes, whereas Cess *et al.* found it to be remarkably constant. Some modellers found this lack of variability in the Cess result even harder to accept than its magnitude, because even when they make their model clouds more absorbing, model-predicted absorption still varies considerably with latitude and other factors.

None of these authors^{1–5} offers any theoretical explanation for enhanced absorption, although Li *et al.* speculate that there may be no mystery absorber and that, instead, algorithms used to process ERBE satellite data may be wrong for highly heterogeneous cloud scenes, such as in the Shuttle photograph shown here (Li's, Cess's and Ramanathan's analyses depend heavily on ERBE, NASA's 1985–1990 Earth Radiation Budget Experiment). Such scenes, with towering convective clouds, are more prevalent in warm seasons, the very seasons where Li *et al.* find the anomaly. And it must be admitted that our understanding of solar

radiation reflected from such cloud scenes is poor. However, ERBE was the result of a gargantuan effort by NASA and universities, and if ERBE algorithms were wrong in the tropics, it would be just as stunning as if cloudy columns contain some elusive mystery absorber.

Li *et al.* also speculate that highly absorbing aerosols from biomass burning cause enhanced tropical absorption. However, tropical biomass burning occurs during a two-month period, and the re-

solar absorber, water vapour, has been revised several times in the past 20 years, and it may not be right yet — especially the all-important continuum component, about which virtually nothing is known.

Anomalous cloudy-column absorption was already being reported in the 1950s⁷. Early measurements were easy to ignore for a variety of reasons, but as the experiments got better, reports of unexpectedly high absorption not only persisted, but were reinforced by observed deficits in near-infrared (wavelength 0.7 to 3 micrometre) cloud reflectivity⁷. Meanwhile, however, other careful observations showed no disagreement with models. Recent measurements by Hayasaka and



Typical heterogeneous cloud field in the tropics, from NASA Shuttle flight STS-035. Such irregular cloud formations may lead to difficulties in processing satellite radiation measurements. (Courtesy of NASA Johnson Space Center.)

sulting aerosols are washed out of the atmosphere less than a month after burning ceases. As the enhanced absorption is seen in every month, this explanation seems unlikely.

Did this challenge catch the field of atmospheric solar radiation napping? Yes and no. The field was wide awake in developing applications (mainly in remote sensing and climate modelling), but napping in basic research. Most practitioners assumed that the current list of atmospheric absorbers was complete and well understood, and that it was safe to use one-dimensional models (which include vertical but no horizontal variation) rather than full three-dimensional models. Model testing in the atmosphere was perfunctory, with little effort to measure model input variables definitively. Now and then there were wakeup calls, but these were quickly forgotten. Even nagging anomalies in clear-sky absorption observations, as well as huge disagreements among models⁶, have remained largely unresolved. The near-infrared absorption spectrum of the dominant

co-workers⁸ recapitulate, in microcosm, this history: in some cases models correctly simulated near-infrared cloud reflectivity observations, in some cases they didn't. The models were correct when the clouds 'looked' more homogeneous, Hayasaka *et al.* said. But even very homogeneous-looking clouds have large turbulent variations in the column amount of liquid water⁹ that are poorly discernible by eye; there is really just a continuum of degrees of inhomogeneity, and it is hard to see how there could be an inhomogeneity threshold past which models suddenly fail.

Fortunately, the United States ARM (Atmospheric Radiation Measurements) programme has planned month-long aircraft campaigns to test the hypothesis of enhanced absorption. These are scheduled at the ARM Oklahoma site this fall, and again in the spring, using the unmanned aeroplanes pioneered by ARM as research platforms. They will eliminate many of the unavoidable uncertainties in comparing satellite and surface radiation measurements; for example, identical high-quality radiometers will be used on

all aircraft and at multiple sites on the surface. And better spectral resolution will pin down which spectral regions are doing the absorbing, something which the satellite and surface radiometers used by Cess's and Li's groups couldn't tell us.

'Enhanced cloud absorption' is a difficult pill for the atmospheric solar radiation field to swallow. Its theoretical models, on which large application programmes in remote sensing and climate modelling are based, have been impugned. Now it must backtrack and fill gaping potholes in fundamental knowledge, including: unscrambling the effects of three-dimensional inhomogeneity from true absorption effects; doing spectroscopy in real and artificially generated clouds; and finding out why shortwave models cannot even agree with one another. Accelerated development of promising new tools to map out the inter-

nal water structure of clouds is needed, to provide input to three-dimensional radiation modelling. Only with much greater attention to such basics can we begin to close this gaping uncertainty in current models. □

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LEUKOCYTE ADHESION

Missing link in angiogenesis

Napoleone Ferrara

A FUNDAMENTAL feature of inflammation is the orderly adhesion of white blood cells to the endothelium of postcapillary venules and their subsequent ingress into affected tissues. Leukocyte recruitment may be followed by the formation of new blood vessels (angiogenesis), which happens in a variety of inflammatory disorders including rheumatoid arthritis. In this disease, the new blood vessels play a central pathogenic role as they confer on the inflamed synovium (pannus) the ability to proliferate rapidly and destroy the articular cartilage¹. Until now, leukocyte/endothelial adhesion and angiogenesis were often considered to be separate processes.

But the discovery of a molecular link is described on page 517 of this issue²: Koch *et al.* report that two mediators of leukocyte/endothelium adhesion, E-selectin and vascular cell adhesion molecule-1 (VCAM-1), when tested in their soluble forms, are potent inducers of endothelial-cell chemotaxis *in vitro* and angiogenesis in an *in vivo* model, the rat cornea. They also show that a large portion of the chemotactic and angiogenic activity of synovial fluid derived from patients with rheumatoid arthritis can be neutralized by specific antibodies against E-selectin or VCAM-1. Previous work had shown that the levels of both adhesion molecules are significantly raised in rheumatoid synovial fluid.

Two distinct sets of adhesive, receptor/ligand-like interactions between white blood cells and vascular endothelium have been identified. One involves the integrin family (for example, VLA4) and their

ligands (for example, VCAM-1)³; the other involves lectin-containing molecules known as selectins, and their carbohydrate ligands immobilized on mucin-like scaffolds⁴. The frequent existence of multiple ligands for each receptor emphasizes the molecular complexity and potential biological diversity of such interactions⁴. Many agents have been identified as mediators of angiogenesis⁵, including interleukin-8, TNF- α and the growth factors aFGF, bFGF, VEGF, angiogenin, TGF- α and - β , and HGF.

An earlier report suggested a role for E-selectin and its ligand sialyl Lewis-X/A in bovine capillary morphogenesis *in vitro*⁶, but so far any link between leukocyte/endothelial adhesion and angiogenesis has remained hidden. The findings described by Koch *et al.*² are important not only because they identify E-selectin and VCAM-1 as potential mediators of the two processes, but also because they provide the first evidence for their involvement in the neovascularization associated with a major inflammatory disorder such as rheumatoid arthritis.

The authors propose the following sequence of events: after binding of leukocytes to vascular endothelial cells, VCAM-1 and E-selectin expressed on the cell surface of endothelial cells are cleaved and shed in a soluble form and are then free to activate and recruit adjacent endothelial cells. Koch *et al.* also explore the mechanism of the endothelial cell chemotactic action of E-selectin and VCAM-1 and show that such an effect is largely mediated by their conventional ligands, sialyl Lewis-X and VLA-4, respectively.

A recent study demonstrates that one of the possible ligands for E-selectin is a variant of a receptor for fibroblast growth factor (FGF)⁷, a known angiogenic mediator⁵. This finding suggests that E-selectin has the potential to participate in the signal transduction pathways of a conventional angiogenic factor and emphasizes the remarkable biological versatility of the selectin family.

An important question is: do soluble adhesion molecules fully account for the neovascularization that takes place in rheumatoid arthritis? The answer is almost certainly no, considering that rheumatoid synovial fluid contains large amounts of several 'classical' angiogenic cytokines. In particular, previous work^{8–10} has shown that interleukin-8, TNF- α , VEGF and HGF, produced primarily by macrophages and synovial-lining cells, may contribute significantly or even substantially to endothelial-cell recruitment and angiogenesis in rheumatoid arthritis. A possible key to this perplexing redundancy is that the actions of these various agents on the endothelium may be, at least in part, temporally and spatially segregated. For example, endothelial-cell-derived E-selectin, which is expressed very early in the inflammatory process⁴, may be important in triggering angiogenesis in the initial stages of the disease when neutrophil adhesion is prominent. In contrast, angiogenic factors released by macrophages or synovial lining cells are likely to be important in maintaining angiogenesis in more advanced phases of the disease.

The findings described by Koch *et al.*² add to our understanding of the complexity of the regulation of angiogenesis. Validation of these findings would be warranted in animal models of arthritis, where their significance could be investigated free of the high variability inherent in human specimens. Also, it would be interesting to know whether the same mechanism operates in other clinically important conditions such as wound repair and tumour growth, in which leukocyte adhesion and angiogenesis are temporally related. □

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Inside the MADS box

Richard Treisman

In a classic experiment, Brent and Ptashne established that the transcriptional activation and DNA-binding functions of the yeast transcription factor GAL4 are encoded by separate protein domains¹. However, many DNA-binding domains do more than just bind DNA. An instructive example is that of the mammalian transcription factor SRF and its close yeast relative MCM1, two proteins involved in extracellular signal-regulated and cell-type-specific transcription². The DNA-binding domains of these transcription factors also interact with accessory proteins to form functionally distinct

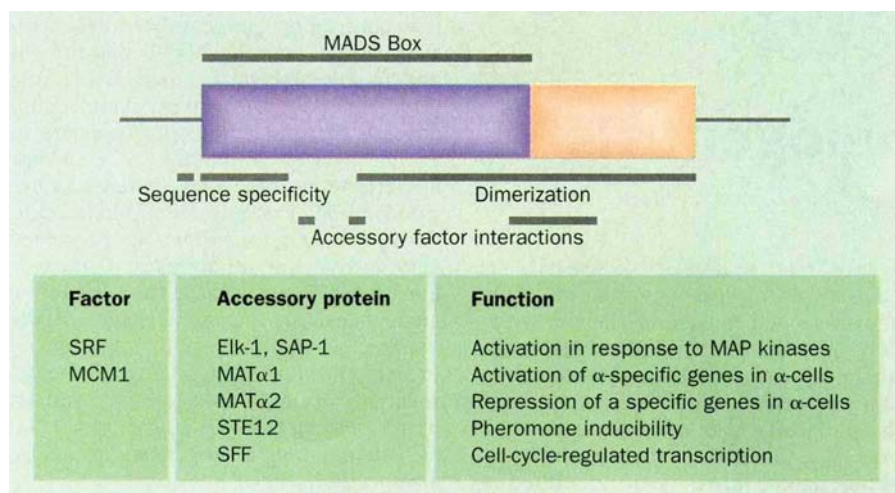
MADS box². The carboxy-terminal half of the MADS box forms part of the dimerization surface; however, efficient dimerization requires an additional 30 residues C-terminal to the MADS box, which are also important for recruitment of accessory proteins⁸⁻¹⁰.

The SRF dimer has a stratified structure in which each of three structural units, stacked one above the other in the monomer, interacts with the same unit in its partner. At the base of the structure is a long antiparallel coiled coil, formed by the interaction of two long amphipathic α -helices encoded by the highly conserved

A·T basepairs and by distamycin binding, both of which alter the structure of the minor groove (S. Natesan and M. Gilman, personal communication). The coiled coil formed by the MADS-box helices sits almost parallel to and on top of a narrowed DNA minor groove, making extensive contacts with the phosphate backbone as suggested by previous mutational analysis¹². Bending of the DNA towards the protein allows the N-terminal part of each helix and the overlying β -sheet to make groove contacts at the edge of the consensus motif, leaving the major groove in the centre of the consensus essentially unobstructed, which might allow simultaneous interaction of the complexes with other proteins, such as the homeodomain protein Phox1 (ref. 13).

The MADS-box sequences N-terminal to the helix duck under the coiled coil, allowing the insertion of a highly conserved arginine side chain into the DNA minor groove. It is this region that is responsible for the differential sequence specificity of SRF, MCM1 and MEF2A (refs 14,15), and this can now be at least partially rationalized in structural terms⁷. Substitution of all SRF sequences N-terminal to the MADS box with a single methionine residue, as in MEF2, alters the binding specificity of SRF to that of MEF2, YTA(T/A)₄TAR; this may arise from hydrophobic methionine–adenine interactions in the minor groove. In contrast, substitution of the N-terminal glycine of the MADS box by glutamate, as in MCM1, alters binding specificity to that of MCM1, CCYAATNNGG, probably by affecting the way in which the side chain of the neighbouring arginine is inserted into the DNA minor groove. It is likely that DNA in the MCM1 and MEF2 complexes may exhibit structural differences from that of SRF, including differences in minor groove width and DNA bending.

The residues involved in the interaction with the various SRF and MCM1 acces-



Simplified schematic representation of the SRF and MCM1 DNA-binding domains. The DNA-binding domain is shown as a box, with the MADS box in purple. Regions of the domain shown biochemically to determine DNA-binding specificity, and to mediate dimerization and accessory factor interactions are indicated. Below are listed the various accessory proteins which form stable complexes with either SRF or MCM1, together with their functional properties. Specificity of complex formation is determined by the sequences adjoining the SRF or MCM1 binding site, in addition to protein–protein interactions.

transcription factor complexes; moreover, sequence-specific interactions with DNA can both modulate transcriptional activation³⁻⁵ and alter protein conformation⁶. Our understanding of how these 90-amino-acid domains can accomplish such feats in addition to mediating protein dimerization and sequence-specific DNA recognition now has a firm structural basis with the elucidation, on page 490, of the structure of the core SRF DNA-binding domain bound to a consensus SRF-binding site⁷.

The DNA-binding domains of SRF and MCM1 are 70% identical, containing at their amino termini a 57-amino-acid sequence motif which defines the MADS (MCM1-Agamous-Deficiens-SRF) box family of DNA-binding proteins (see figure). Biochemical studies show that different family members bind DNA with related but distinct specificities determined by the N-terminal basic half of the

central section of each MADS box. On top of this sits a four-stranded antiparallel β -sheet, formed by the C-terminal part of each MADS box, above which rests the third component of the dimerization surface, formed by the sequences C-terminal to the MADS box. The layered structure shows how different family members can incorporate widely divergent sequences C-terminal to the MADS box. Moreover, it suggests why SRF does not form homodimers with the MEF2 proteins, a subfamily of mammalian MADS-box proteins which possesses a divergent, presumably structurally distinct, C-terminal region¹¹.

The novelty of the SRF–DNA complex lies in its extensive minor groove interactions and the recognition of SRF-induced conformational changes in the DNA consensus motif. Indeed, binding of SRF to its CC(A/T)₆GG consensus is prevented both by I·C substitution of the central

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sory proteins are mainly clustered towards the top of the protein dimer, where they form a surface that is exposed to solvent. One end of the antiparallel β -sheet that forms part of this region contacts the DNA phosphate backbone adjacent to the consensus motif, suggesting that DNA binding might affect this surface.

The structure also reveals the location of MCM1 mutations that bind certain sites well but exhibit defective activation or impaired interaction with the accessory protein MAT α 1 (ref. 5). These occur within the long MADS-box helix beneath the putative contact surface, close to the DNA, suggesting that they might prevent appropriate DNA-induced conformational changes in the protein. Mutations in both the corresponding regions of SRF can also impair activation from certain

sites, but in this case point mutants have not yet been identified^{3,4}.

The SRF-DNA structure provides a first step towards understanding how the activity of MADS-box proteins is regulated by interaction with DNA and accessory proteins, but much remains to be done. Confirmation of the role of DNA-induced structural changes in transcriptional regulation by SRF and MCM1 will require the elucidation of the interactions between wild-type and mutant proteins complexed with different binding sites. Ironically, it will also be interesting to see what these DNA-binding domains look like in the absence of DNA. $\square$

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OZONE DEPLETION

Methyl bromide under scrutiny

James H. Butler

ATMOSPHERIC methyl bromide (CH_3Br) is believed to be the chief source of stratospheric bromine, which is extremely effective in converting ozone to oxygen. Atom for atom, bromine is about 50 times more effective than chlorine in destroying stratospheric ozone¹; reactions involving bromine are believed to be responsible for 20–25% of the Antarctic 'ozone hole' that develops each austral spring². Mainly because of this efficiency, CH_3Br has been designated as a controlled ozone-depleting substance by international agreement³. Some countries, such as the United States and Denmark, have already taken action to phase out the production and import of CH_3Br ; others are considering such restrictions. But much remains unclear about the origins and action of CH_3Br . Sessions at two meetings held this summer* aimed to iron out some of the uncertainties.

Because the lifetime of CH_3Br is less than 2 years, cessation of emissions should produce prompt results. The benefit of such action could be realized in a few years, whereas for the longer-lived chlorofluorocarbons (CFCs), it would take until the year 2050 to reduce concentrations to what they were before the ozone hole developed. It will take centuries to reduce the atmospheric burden of the CFCs by a factor of 1,000; the atmospheric burden of anthropogenic CH_3Br could be reduced by that much in less than a decade.

Methyl bromide exists in today's atmosphere at a mole fraction of around 10 parts

per trillion (1 p.p.t. = 10^{-12}), an amount that could make it almost as effective as some CFCs in depleting stratospheric O_3 . But the case for restricting its use is not so clear-cut. Unlike the CFCs, CH_3Br is not entirely anthropogenic in origin. Its known sources include oceanic emissions, biomass burning, agricultural application, automobile emissions from leaded gasoline and fumigation. Of these, oceanic emissions and a fraction of the biomass burning emissions are natural. Of the total global emissions of 100–200 Gg yr^{-1} (ref. 1), between 30 and 65 Gg yr^{-1} are believed to be emitted during fumigation. Adding this to emissions of 0.5–22 Gg yr^{-1} from the conversion of ethylene dibromide during combustion of leaded gasoline, the total emission of CH_3Br from industrially produced compounds could be 20–65% of the total budget.

Natural emissions are complicated by the dual role of the ocean — it is both the largest source and the second-largest sink of atmospheric CH_3Br — and by the distribution, origin and relative emissions of wildfires. It is against this backdrop that scientists met to discuss the sources, sinks and stratospheric chemistry of CH_3Br and other related gases. The AGU Special Session dealt with the aquatic production, degradation and emission of a number of gases, including CH_3Br . The Monterey Workshop aimed to update our understanding of atmospheric CH_3Br , identify remaining uncertainties and aid coordination of research.

The budget of atmospheric CH_3Br is reasonably constrained by current estimates of the atmospheric burden, lifetime and interhemispheric gradient; any description of the system must fit within this

framework. The biggest issues facing scientists in the field are, first, balancing the sources and sinks while accounting for the observed interhemispheric gradient; second, determining how much anthropogenic emissions contribute to the total atmospheric budget; third, assessing the relative contribution of CH_3Br to total stratospheric bromine; and, finally, evaluating the efficiency of bromine in depleting stratospheric ozone.

Lifetime estimates with respect to CH_3Br loss in the atmosphere⁴ and to the ocean have improved considerably in the past year (S. Yvon, National Oceanic and Atmospheric Administration, Climate Monitoring and Diagnostics Laboratory (NOAA/CMDL); T. Tromp, Atmospheric and Environmental Research, Massachusetts). Nonetheless, the sources and sinks are still not reconciled. Much of the problem lies in quantifying and mapping the oceanic source. Data from the Pacific and Atlantic Oceans show the consequences of simultaneous production and degradation in the ocean⁵: CH_3Br is undersaturated throughout most of the open ocean, but near equilibrium with the atmosphere in areas of upwelling, and supersaturated in coastal waters. CH_3Br must still be produced in regions where it is undersaturated, as the observed concentrations are insufficient to support known chemical losses, which can reach 3% per day in tropical waters (D. King, Univ. Miami).

Aquatic production of CH_3Br is most probably biological, yet so far no organism (or group of organisms) has been identified that can account for the oceanic emissions (about 60 Gg per year) required to support observed saturations. At the Baltimore meeting, seaweeds were implicated clearly in the production of numerous halomethanes, but their global distribution does little to explain CH_3Br emissions from the open ocean. Ice algae (mostly diatoms) apparently produce little CH_3Br (W. T. Sturges, Univ. East Anglia). Planktonic diatoms typical of temperate to polar latitudes may produce CH_3Br , but not fast enough to support estimated oceanic emissions (R. M. Moore, Univ. Dalhousie). Bacteria have not been found to be involved, although CH_3Br production apparently continues during decay of plant cultures.

The two other large, known sources of atmospheric CH_3Br are anthropogenic emissions and biomass burning. CH_3Br is widely used to fumigate soil before planting fruit and vegetables. Deeper application, and use of tarpaulins to lengthen the residence time in the soils, apparently reduce emissions to the atmosphere (J. Williams, Univ. California at Irvine). Relative emissions also seem to depend on the soil's acidity, temperature and water content, among other properties⁶; some organisms degrade CH_3Br following

*Special Session on Production and Fate of Atmospheric Organic Halogens in the Marine Environment, Spring Meeting of the American Geophysical Union, Baltimore, Maryland, 30 May–2 June 1995; 1995 Methyl Bromide State of the Science Workshop, Monterey, California, 5–7 June 1995.

fumigation (L. G. Miller, US Geological Survey). Given these factors, it is clear that more study is needed before estimates of relative emissions from soil can be improved beyond the current range of 20–85% of the amount applied. The most recent studies of biomass burning emissions lower the estimate from the 30 Gg yr⁻¹ given in the WMO Scientific Assessment to a probable value of 15–25 Gg yr⁻¹, with no significant emphasis in either hemisphere (D. Blake, Univ. California at Irvine; J. E. Penner, Lawrence Livermore National Lab.).

Until recently, it was generally believed that the only important sinks for atmospheric CH₃Br were reaction with tropospheric OH and irreversible loss to the ocean. But new investigations show soil degradation rates which, when extrapolated globally, represent a large, natural sink (C. E. Kolb, Aerodyne, Maryland; P. M. Crill, Univ. New Hampshire). Apparently due to deposition and rapid biological degradation, this process creates a 42 (±32) Gg yr⁻¹ (25%) disparity in the budget. It is also difficult to reconcile with the interhemispheric ratio (north/south) of 1.3 for CH₃Br in the atmosphere, which requires that net emissions in the Northern Hemisphere are double those in the Southern Hemisphere. As the soil sink is likely to be stronger in the north than in the south, the new findings require an equivalent weighting of sources in the Northern Hemisphere, something that is difficult to do in current models.

Estimates of the strengths of all three sinks depend on an accurate determination of the atmospheric burden. Recent intercalibrations between NOAA/CMDL and Scripps Institute of Oceanography suggest that the present atmospheric mole fraction of CH₃Br is about 10% lower than that given in the recent WMO Scientific Assessment (S. A. Montzka, NOAA/CMDL; R. F. Weiss, Scripps Inst. Oceanography). This helps to balance the budget by reducing required sources. Further intercalibrations among laboratories would be valuable. Also, it was pointed out at both meetings that analyses of stored air are often impaired by growth of CH₃Br in the containers, even over a short time, so new measurements of archived air may not reliably detect growth of atmospheric CH₃Br over the past two decades when agricultural uses were increasing, leaded gasoline consumption declining, and atmospheric measurements limited. Recent atmospheric measurements have not spanned sufficient time to detect trends.

Once delivered to the lower stratosphere, CH₃Br is rapidly photolysed to inorganic bromine. Unlike chlorine, much of the inorganic bromine in the lower stratosphere is reactive and even the reservoir species are short-lived. This is largely what makes bromine more ef-

ficient at destroying ozone. But there is still uncertainty in the reaction rates of inorganic bromine radicals, particularly for the BrO+HO₂ reaction (G. Poulet, Centre National de la Recherche Scientifique; Z. Li, Jet Propulsion Lab.) and for reactions involving BrONO₂ or HOBr (ref. 7).

In Monterey, investigators reported BrO data from aircraft (ER-2) and balloon-borne instruments. Reports⁸ suggest that BrO abundance in the lower stratosphere ranges from 5 to 8 p.p.t. by volume. This is lower than values calculated with photochemical models that assume total bromine (inorganic plus organic) to be 18–20 p.p.t., and that are consistent with measurements of organic bromine just below the tropopause⁹. ER-2 measurements of BrO from 1994 (R. M. Stimpfle, Harvard Univ.) are higher and predict bromine levels nearer to 25 p.p.t. in the stratosphere. This implies that shorter-lived compounds could be getting into the lower stratosphere through convection of air from the marine boundary layer. Also, measurements taken with the tracer gas SF₆ during ER-2 flights indicate that lower stratospheric air may be less than a year old (J. W. Elkins, NOAA/CMDL). Unfortunately, uncertainties in the measurements of BrO (±30%) and in computations (±80%) are large and more analysis is needed before this issue can be resolved.

As both meetings showed, information on the emission, transport and reaction of atmospheric CH₃Br has been flooding in over the past couple of years, often changing our ideas about the behaviour of this compound in nature. Estimates of the atmospheric burden and lifetime of CH₃Br have improved enormously, as has our understanding of its sources and sinks. Research still continues at a rapid pace. Currently, though, the budget of atmospheric CH₃Br remains uncertain, as do estimates of just how much it contributes to the total destruction of stratospheric ozone. Reducing these uncertainties must lie at the heart of the continuing research effort. □

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DAEDALUS

St Elmo's phone

LAST week Daedalus had a scheme for focusing microwaves high in the air. It exploited the high resolution of a synthetic-aperture receiver made up of widely spaced but interlinked radio telescopes. Daedalus planned to work the whole system in reverse, with the dish aerials of the array operating as coupled microwave transmitters. With correct phasing, the array could concentrate a beam of microwaves at a defined point in space perhaps 10 cm across.

About 100 kW of microwave power, concentrated in a small volume high in the atmosphere, should break down the dielectric resistance of the air, especially if dust or floating particles provided discharge points for an initial corona. The result would be a stable sphere of ball lightning (which, according to one theory, is a spherically symmetrical radio-frequency discharge powered by the intense field gradients of the thundery atmosphere). By changing the phasing of the transmitters in the array, this ball of lightning could be swept around the sky, and raised or lowered on command.

The obvious application is for street lighting, sky-writing and pyrotechnic displays. But Daedalus has higher technology in mind. He recalls an over-the-horizon communication system that bounced its signal off high-altitude meteor tracks. Such tracks can be quite frequent; their hot, ionized air conducts electricity well enough to be a good radio reflector.

So Daedalus wants to establish a stable ball-lightning discharge about 120 km up in the stratosphere, for use as a large-scale communications relay. The microwave beams that maintain it could easily be modulated with telephone, radio, television and digital data. The ionized, conducting ball would reradiate them over an area perhaps 1,200 km in radius. Any other communications system could also beam its signal at the ball and have it reflected evenly over the same area. The ball will probably generate some radio-frequency noise on its own account, but with luck this will be confined to specific bands, or be easy to filter out.

Geosynchronous satellites, with their feeble transmitters, vast expense and limited life, will thus be outmoded. A set of stable ionospheric ball-lightning discharges, closer to Earth and pumping out far more power, will do their job more cheaply and flexibly. The simple receivers needed will be easy to align on the relay, even at extreme range. You will simply look for the bright artificial 'star' glowing fixedly in the night sky, and point the aerial straight at it.

David Jones

Measuring sexual selection

SIR — Many researchers have measured sexual selection on mating arenas (leks) in a wide variety of species. These studies have influenced our understanding of sexual selection because of the extreme variation in mating success among lekking males. One measure of this variation, used recently by Widemo and Owens¹, is the degree of skew. We will show that this measure has drawbacks.

Widemo and Owens examined mating skew on leks of different sizes to understand the decisions made by individuals of different rank. They suggested that for a species of wading bird, the ruff (*Philomachus pugnax*), mating skews decrease in larger leks because of increased aggression. This interesting approach illustrates the difficulties of using skew to measure variation in mating success. A null version of their model in which matings occur entirely at random shows that the mea-

sure of skew should decrease exponentially with lek size (a in the figure). Biological explanations for the drop in skew in larger leks are therefore unnecessary, as the drop can occur as an artefact of any formula for skewness.

This raises the more general issue of how to quantify sexual selection. One reason for the popularity of skew is that data are often depicted in histograms of mating success for individuals that have been ranked along the x -axis according to their success. These are not frequency distributions, but perhaps because of their apparent similarity it is tempting to quantify their asymmetry using measures of skewness from frequency distributions. The measure that best expresses the dispersion of a distribution is the variance, which is independent of sample size and has the considerable advantage of using classical evolutionary genetics to

quantify intensity of selection².

Comparison of male mating distributions involves two important variables: the number of males and the number of matings. Although variance is unaffected by the number of males, both skew and variance are affected by the number of matings. If the mean male mating success changes in a lek of fixed size, there is a negative correlation between the variance in mating success and the skew index, whether mating is random or aggregated (b in the figure).

Under random mate choice, the variance should equal the mean. The extra component of variance, beyond that due to random processes, can thus be determined simply by subtracting the mean from the variance. It is not readily possible to disentangle the skew statistic from its interdependence on both the number of males and the number of matings.

Does variance in mating success generally decline with lek size? Elsewhere³, we have discussed a range of factors contributing to variance, including male quality and female copying. A survey of 74 leks from 19 species shows that across all leks mean male mating success accounts for 88% of the variance (c in the figure). The residual variance is not negatively correlated with lek size (d in the figure). Comparison of multiple leks within species still shows no pattern. In four species the trend was towards higher variance in larger leks, while in one species there was less.

Although we disagree over the measure of skew, we agree with Widemo and Owens that the sensible approach is to look at the costs and benefits to different males of displaying on different-sized leks. Unattractive males may indeed benefit from associating with attractive individuals. In explaining the colonial behaviour of fish, an equivalent approach has been useful⁴.

Aulay Mackenzie

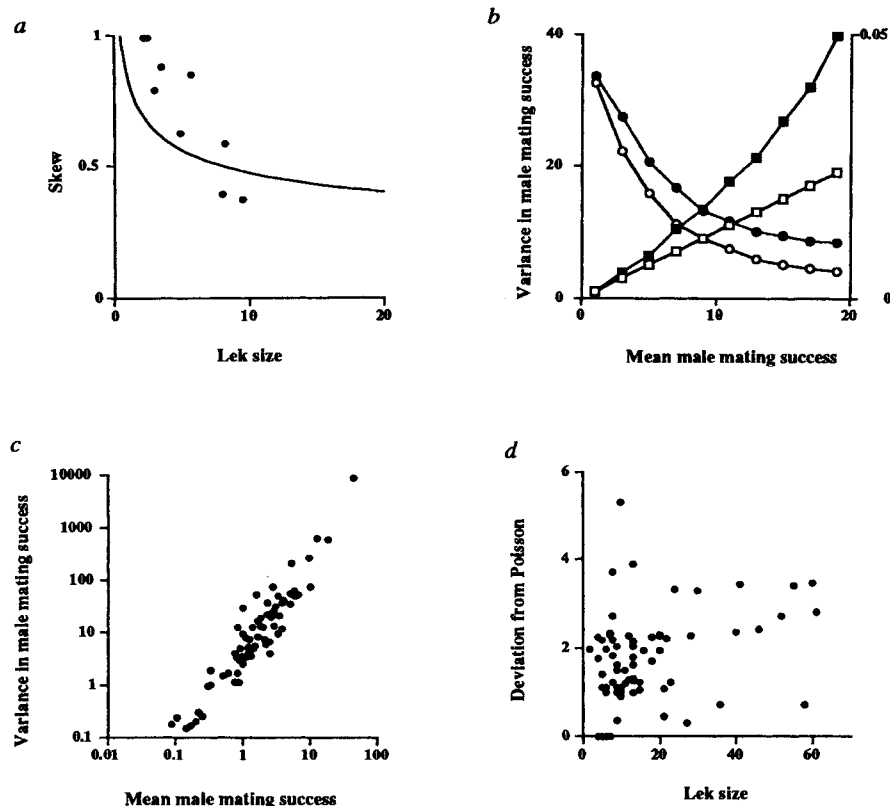
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WIDEMO AND OWENS REPLY — We agree with Mackenzie *et al.* that variance has several convenient properties as a measure of the opportunity for sexual selection, even though Sutherland⁵ has pointed out several serious problems with this approach. We maintain, however, that the skew index used by us handles some situations better than does variance since it takes the relative rather than the absolute differences between males into account.

We did not set out to measure the intensity of sexual selection, however. Our main point¹ was that it is necessary to focus on the success of individual, rather than



a, Relationship between lek size and 'skew index'¹. The points are ruff data¹ and the line is the expected relationship when females select males randomly (the null model). The expected distribution assumes that mean male mating success remains constant at unity throughout and hence the variance in male mating success is unity, although the relationship is not strongly affected by deviations from this assumption. *b*, Relationship between variance in male mating success and the skew index with respect to the mean male mating success (squares, variance; circles, skew; open symbols, random mating; solid symbols, aggregated matings; coefficient of variation² of aptitude for an encounter = 0.1). Lek size is 10. *c*, Relationship between mean male mating success and variance in male mating success for 74 leks of 19 species. All leks show greater variance in male mating success than expected from random mating. A linear regression with a slope significantly greater than unity ($P < 0.001$) explains 88.0% of the variance in the variance of male mating success. *d*, Relationship between lek size and the variance in male mating success above that expected from random mate choice for 74 leks of 19 species, given by: $\log_e(\text{variance in male mating success}) - \log_e(\text{mean male mating success})$. Details of the model, references and species are given in ref. 3.

average, male success when discussing how males should distribute themselves between leks of different sizes. We illustrated the effect of lek size on proportion of copulations accruing to males by calculating a composite skew index for each lek, rather than showing the relationships for each rank of male. This point remains unaffected by the criticism by Mackenzie *et al.* The model as well as all analyses of the empirical data deal solely with proportions of copulations accruing to males of different rank, however. What requires a biological explanation is not the drop in skew, but the fact that males of the same rank get different proportions of the copulations on a lek depending on the lek size.

Mackenzie *et al.* use empirical data to suggest that the conclusions of our paper are unlikely to be general. They justify this by showing that the variation in male mating success not explained by random mating does not covary with lek size in an interspecific comparison. We do not think that this is an adequate test of our model, as the ability of top males to monopolize matings is likely to vary across species. Consequently, the interaction between lek

size and the proportion of copulations accruing to a male of a given rank will vary between species. Also, the variance in mating success generated by random events is likely to differ between species⁵. Hence, we would not expect to find a cross-species correlation between lek size and deviation from a Poisson distribution of copulations. The intraspecific tests described by Mackenzie *et al.* are more interesting, but at this stage are too preliminary to allow evaluation.

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Pasture soils as carbon sink

SIR — Fisher *et al.*¹ draw much-needed attention to the important role of deep tropical soils and tropical land use in the global carbon cycle. They show that African forage grasses planted in South American cattle pastures have prolific root systems extending below the plough layer. They calculate that the enhanced soil carbon storage resulting from increased root inputs may be large enough to be the 'missing sink' needed to balance the global carbon budget². Hence, the conversion of savannas to cattle pastures is presented as a win-win situation, because excellent sources of forage for cattle also purportedly reduce accumulation of heat-trapping CO₂ in the atmosphere. We believe that Fisher *et al.* have overestimated the potential for pasture soils to be a significant carbon sink, and we also point out the deleterious effects of introduced African grasses owing to their

tendency to invade native savanna vegetation and their high flammability.

First, the results of Fisher *et al.* pertain only to savanna areas that lack significant woody vegetation; this excludes most of the Brazilian cerrado and the Amazonian rainforests, which are also being cleared and converted to cattle pastures with exotic grasses. These ecosystems lose carbon on conversion because of the loss of significant above-ground biomass.

Second, Fisher *et al.* have assumed that their measured increases in soil carbon during the 3–9 years after pasture creation would continue indefinitely. This extrapolation is incorrect, because rates of soil microbial respiration will also increase as organic matter accumulates. Because the higher carbon inputs from pasture grasses are matched by increased microbial decomposition, the soil will eventually approach a new steady-state carbon inven-

tory. The rate of net carbon accumulation is greatest for the first few years after an increase in carbon input, but approaches zero at steady state. The time required to establish steady state (or near steady state) depends on the decomposition rate of soil organic matter. Using ¹⁴C from the fallout of nuclear weapons testing as a tracer, it has been shown that most of the carbon in the top metre of tropical soils has a mean residence time of up to a decade^{3,4}. Under these conditions, the annual rate of net carbon accumulation after 20 years will drop to about 10% of the net carbon accumulation observed during the first three years following pasture establishment. A sustained carbon sink of the magnitude suggested by Fisher *et al.* would require continued establishment of new pasture, as the net carbon sink associated with older pastures declines.

Third, bad management and poor productivity of African grasses are common in much of South America. The studies by Fisher *et al.* come from experimental stations where production is presumably optimized. Many (and perhaps most) pastures planted with *Brachiaria* in both forested⁵ and savanna⁶ regions of Brazil are in some stage of degradation, usually due to overgrazing, extensive use of fire, invasion by unpalatable species and soil compaction. Degraded pastures typically have little grass cover and low inputs of carbon to the soil⁷. Unfortunately, too few data exist to estimate quantitatively how much pasture land is being managed well. To extrapolate a carbon sink across 35 million hectares of South American pastures, however, Fisher *et al.* need to provide some evidence that good pasture management is common over that large area. In contrast, several lines of evidence indicate that less than optimal grass yield is common, including reports of loss of nutritional value of African grasses during the dry season⁸, failure to maintain a legume association^{6,9} and infestations of spittlebug¹⁰.

Finally, increased use of African grasses will result in further biotic impoverishment of ecosystems. Multi-species communities are being replaced by monocultures of exotic grasses. The remaining native savannas are also threatened by invasion of exotic species well adapted to displacing native grasses, through their rapid growth rate, high seed production, fast germination and colonizing ability^{11–13}. In addition to the increased probability of intentional fires escaping from managed pastures, the exotic species that invade nearby, native savanna are highly flammable and burn at a higher temperature than native grasses¹³, potentially altering the frequency, intensity and extent of fire. We can thus expect functional as well as compositional change to these ecosystems.

We acknowledge that exotic grasses may offer promise by increasing productivity in

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many tropical pastures. If productivity can be sustained, the high economic reward per unit area of well-managed pastures might reduce demand for large-scale clearings of forests and savannas to meet local agricultural and economic needs. A globally significant carbon sink is, however, unlikely to be a virtue of this land use and cannot be used to justify it. Moreover, the effect of invading exotic species on native vegetation must also be considered before singing the praises of this new trend.

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FISHER *ET AL.* REPLY — The comments by Davidson *et al.* about the rainforests and the wooded communities of the cerrados are not relevant to our paper¹, which was about the 35 million hectares (MHa) of treeless grasslands in Colombia and Venezuela and the 50 MHa (24%) of the cerrados of central Brazil that have no significant woody component (*Campo limpo* and *Campo sujo*¹⁴). It is on the latter, which have less fertile soils, that most of the 35 MHa of introduced pastures¹⁵ have been sown in the past 30 years. The plant communities of the cerrados with a significant tree component are on more fertile soils, and when cleared have normally given way to cropping, largely because the economics of cattle production will not support the high cost of mechanical clearing.

We did not forecast that the rates of C sequestration will continue indefinitely. We measured rates of C sequestration of 2.9–14.7 t Ha⁻¹ yr⁻¹ in the soil under pastures of introduced grasses compared with the native savanna on a farmer's fields and at Carimagua research station in the eastern plains of Colombia. We hypothesized that if this process is general in sown pastures in the neotropical savannas, then the amount of C sequestered could be large enough to be important.

We do not yet know the dynamics of C in the soil under our pastures. In the same samples from Carimagua that we reported in our paper, not only are the C:N ratios of the soil under savanna unusually high at 21.5, but also we measured a shift to 33.2 after nine years of introduced grass pasture, five of them with a legume (s.e. of difference ± 6.57 , $n=7$, $P<0.001$). For this to occur, the C:N ratio of the newly accumulated soil organic matter must be very high. We do know that the C:N ratios of litter of the African grasses are unusually

high. For above-ground litter of *Brachiaria decumbens*, *B. dictyoneura* and *B. humidicola* and *Andropogon gayanus* they are 74.8–193.5 (ref. 16), and for fine and coarse roots of *B. dictyoneura* and *B. humidicola* they are 158 and 224 (ref. 17). It would be dangerous to apply conventional wisdom to organic matter derived from this litter because it is likely to be less easily broken down by soil biota, and therefore the soil C may have longer residence times. We accept that there will be a new equilibrium, but at what level and when is an open question.

With regard to management, we have measured C sequestration of 2.9 t Ha⁻¹ yr⁻¹ in a 17-yr-old pasture of *A. gayanus* that had been subjected to mismanagement by burning, over- and undergrazing at least as bad as the worst farmers' fields. This rate is the same as in pure grass pastures reported in our paper, whereas our data also show that well-managed pastures with a good legume balance can sequester C at up to five times this rate.

All grasses lose some nutritional value during the dry season, but the introduced ones at all times have higher quality than the savanna species they replace¹⁸. This, and their deep-rootedness, which allows them to grow longer into the dry season, are the main reasons farmers have sown 35 MHa in the Brazilian savannas¹⁹. There have been some problems with persistence of tropical legumes sown with introduced grasses, in part due to their different photosynthetic pathways²⁰. But *Arachis pintoi*, the legume in one of our experiments, has persisted in mixture with contrasting grasses in the Colombian Llanos under differing managements for as long as 13 years²¹. The Brazilian experience is shorter, but there are no recorded failures once the legume is well established. Spittlebug has been a problem in some *Brachiaria* spp. in some humid areas of the neotropical savannas.

Conserved cell and organelle division

SIR — The process of organelle division in eukaryotes is poorly understood and no genes involved in this process have yet been isolated. In prokaryotes, from which both chloroplasts and mitochondria probably evolved¹, several genes essential for cell division have been identified. The best characterized encodes the protein FtsZ, which forms a ring at the leading edge of the cell division site². It has been proposed that FtsZ is a prokaryotic cytoskeletal element and possibly an evolutionary progenitor of tubulin³. The role of FtsZ in prokaryotic cell division suggested to us that a similar protein might be involved in the division of eukaryotic organelles.

We used the amino-acid sequence of *Escherichia coli* FtsZ as a probe in a homology search of the Expressed

Breeding for resistance to it in *Brachiaria* spp. is a major objective of plant improvement both at CIAT and by the Empresa Brasileira de Pesquisa Agropecuária (EMBRAPA) in Brazil²².

We share the concern about the conservation issues raised by Davidson *et al.*, but to address them was outside the scope of our paper. *Panicum maximum*, *Hyparrhenia rufa* and *Melinis minutiflora*, African grasses introduced to the neotropics almost 100 years ago, do invade native savanna¹¹, particularly in the absence of fire. We have looked for, but not found, invasion of undisturbed savanna by *A. gayanus* or *Brachiaria* spp., the species in our experiments and the main ones sown over the past 20 years in the neotropics.

A large portion of the neotropical savannas has been replaced by sown pastures and crops for economic and other reasons. Our point is not that savannas and grasslands ought to be replaced by introduced pastures, but that, when they are, the pastures can have beneficial effects in terms of C sequestration in the soil.

We agree with Nepstad *et al.*⁷ that there is a need to understand the dynamics of soil–plant processes associated with C sequestration in tropical American soils. We would extend this to include the introduction, degradation and reclamation of pastures based on African grasses in tropical America and the trade-offs involved.

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Sequence Tag database dbEST⁴. Complementary DNA from *Arabidopsis thaliana*, with no assigned matches in the database but exhibiting a small stretch of homology to *E. coli* FtsZ, was identified, obtained from the Arabidopsis Biological Resource Center, and sequenced fully. The open reading frame encoded a protein of 433 amino acids (M_r 45,600) with significant homology to FtsZ sequences from several prokaryotes (Fig. 1). Noteworthy in the *Arabidopsis* sequence are conservation of the glycine-rich 'tubulin signature' motif which is common to both FtsZs and tubulins and is important for GTP binding⁵. All but one of the residues identical in bacterial FtsZs and tubulins⁶ are also conserved.

The *Arabidopsis ftsZ* gene seems to be most closely related to the prokaryotic

<i>Arabidopsis</i>	MAIIPLAQLN	ELTISSSSSS	FLTKSISSHS	LHSSCICASS	RISQFRGGFS	KRRSDSTRSK	60
<i>Anabaena</i>		MTLDNNQELT	YRNSQLGQP	GFSLAVNSSN	PPNHSGLNFG	QNNDSKKSIV	50
<i>B. subtilis</i>					MLEFE	TNIDG.....	10
<i>S. aureus</i>					MLEFE	QGFNH.....	10
<i>E. coli</i>					MFEPH	EL.....	7
<i>R. meliloti</i>					MTEYK	KPIIT.....	10
<i>Consensus</i>							
<i>Arabidopsis</i>	SMRLRCFSFP	MESARIKIVG	VGGGGNNAVN	RMISSGLQSV	DFYAINTDQ	ALLQFSAENP	120
<i>Anabaena</i>	ENNRIGEIVP	GRVANIKVIG	VGGGGNNAVN	RMIESDVSGV	EFWSINTDAQ	ALTLGAPSR	110
<i>B. subtilis</i>		..LASIKVIG	VGGGGNNAVN	RMIEVEVQGV	EYIAVNTDAQ	ALNLSKAEVK	58
<i>S. aureus</i>		..LATLKVIG	VGGGGNNAVN	RMIDHGMNIV	EFIAINTDQ	ALNLSKAEVK	58
<i>E. coli</i>		TNDAVIKVIG	VGGGGNNAVE	HMVRIEIEGV	EFFAVNTDAQ	ALRKTAVGQT	57
<i>R. meliloti</i>		EMRPKITVIG	VGGGGNNAIN	NMIAENLQGV	DFIAANTDAQ	ALATSKAERR	60
<i>Consensus</i>		VIG	VGGGG NA	M V	NTD Q	AL	
<i>Arabidopsis</i>	LQIGELLTRG	LGTGNNPLLG	EQAAEESKDA	IANALKGSGL	VFITAGMGGG	TGSGAAPVVA	180
<i>Anabaena</i>	LQIQQLTRG	LGAGNPAIG	QKAAEESRDE	IATALEGADL	VFITAGMGGG	TGTGAAPIVA	170
<i>B. subtilis</i>	MQIGAKLTRG	LGAGANPEVG	KKAAEESKEQ	IEEALKGADM	VFFVTAGMGGG	TGTGAAPVIA	118
<i>S. aureus</i>	IQIGELKTRG	LGAGANPEIG	KKAAEESREQ	IEDAIQAGDM	VFFVTAGMGGG	TGTGAAPVIA	118
<i>E. coli</i>	IQIGSGITKG	LGAGANPEVG	RNAADEDRDA	LRAALEGADM	VFLAAGMGGG	TGTGAAPVVA	117
<i>R. meliloti</i>	IQLGAAITKG	LGAGSVDPDG	NAAQESIDE	IMDHGEGTEM	CFVTAGMGGG	TGTGAAPVIA	120
<i>Consensus</i>	Q G T G L G G P G		AA E	G F	GMGGG	TG GAAP A	
<i>Arabidopsis</i>	QISKDAGYLT	GVGVTPPFSF	EGRKRLQAL	EAIEKLQKNV	DTLIVIPNDR	LLDIADGETP	240
<i>Anabaena</i>	EVAKEMGALT	GVGVTRPFPF	EGRRTSQAR	QGIEGLKSRV	DTLIIIPNNK	LLEVIPEQTP	230
<i>B. subtilis</i>	QIAKDLGALT	GVGVTRPFPF	EGRKRLQAL	QGIEGLKSRV	DTLIVIPNDR	LLEVIPEQTP	178
<i>S. aureus</i>	KIAKEMGALT	GVGVTRPFPF	EGRKRLQAL	AGVEMAKAAV	DTLIVIPNDR	LLDIVDKSTP	178
<i>E. coli</i>	EVAKDLGILT	VAVVTKPPNF	EGRKRLQAL	QGIEGLKSRV	DTLIVIPNDR	LLKVLGRGIS	177
<i>R. meliloti</i>	EAARAGILT	VAVVTKPPNF	EGRKRLQAL	LGVERLRESA	DTLIVIPNDR	LFRIADAKT	180
<i>Consensus</i>	G LT V VVT PF F	EG R A		D I IPN			
<i>Arabidopsis</i>	LQDAFLADD	VLRQGVQGIS	DIITIPGLVN	VDFADVKAVM	KDSGTALMGV	GVSSSKNRAE	300
<i>Anabaena</i>	VQEAFLYADD	VLRQGVQGIS	DIITIPGLVN	VDFADVRVAV	ADAGSALMGV	GVSSGKSRAE	290
<i>B. subtilis</i>	MLEAFREADD	VLRQGVQGIS	DLIATPGLIN	LDFADVKTIM	SNKGSALMGV	GIATGENRAA	238
<i>S. aureus</i>	MMEAFKEADD	VLRQGVQGIS	DLIATPGLIN	LDFADVKTIM	SNKGSALMGV	GVSSGENRAV	238
<i>E. coli</i>	LLDAFGAAND	VLRQGVQGIS	ELITRPGLMN	VDFADVKTIM	SEMGMAMGVS	GVASGEDRAE	237
<i>R. meliloti</i>	FADAFMIADR	VLYSGVSCIT	DLIVKEGLMN	LDFADVKTIM	KGMGRAMMGT	GEATGENRAE	240
<i>Consensus</i>	AF A VL V I	I G N	DFADV	M G A G G		RA	
<i>Arabidopsis</i>	EAAEQATLAP	LI.GSSIQSA	TGVVYNITGG	KDITLQEVNR	VSQVVTSLAD	PSANIIFGAV	359
<i>Anabaena</i>	EAAIAAISPP	LL.ECSIEGA	RGVVFNITGG	SDLTLEHENA	AAETIYEVVD	PNANIIFGAV	349
<i>B. subtilis</i>	EAAKKAISPP	LL.EAAIDGA	QGVLMNITGG	TNLSLYEVEQ	AADIVASASD	QVNMIFGVS	297
<i>S. aureus</i>	EAAKKAISPP	LL.ETSIVGA	QGVLMNITGG	ESLSLFEAQE	AADIVQDAAD	EDVNMIFGVS	297
<i>E. coli</i>	EAAKKAISPP	LL.EEDIDLSGA	RGVLFVNITAG	PDLLRDEFET	VGMTIRAFAS	DNATVIVIGTS	297
<i>R. meliloti</i>	LAAEAAIANP	LLDEVSMRGA	KGVLVISISGG	MDMTLFEVDE	AATRIREEVY	DEADIVVGAI	300
<i>Consensus</i>	AA A P L	A G V I G	L E		G		
<i>Arabidopsis</i>	VDDRYTGEIH	VTLIATGFSQ	SPQKTLTLPD	RAA..KLLDK	MGSSGQQENK	GMSLPHQKQS	417
<i>Anabaena</i>	IDDRLQGEVR	ITVIATGFTG	..EIQAAPQ	NAANARVVA	PPKRTPTQTP	LTNSPAPTEP	407
<i>B. subtilis</i>	INENLKDEIV	ITVIATGFTG	..EKDVTKPQ	NAANARVVA	PPKRTPTQTP	LTNSPAPTEP	353
<i>S. aureus</i>	INPELQDEIV	ITVIATGFTG	KPTSHGRKSG	STGFTGVTPT	SSNATSKDES	FTSNSSNAQA	357
<i>E. coli</i>	LDPDMNDEL	ITVATGFI.G	MDKRPRITLV	T..NKQVQNP	VMDRYQQHGS	APLTQEQKPV	354
<i>R. meliloti</i>	FDRSLDGTFR	VS.VATGL.D	SNRSQAQTPA	EAMNGQTAAA	VPSRTQL...		345
<i>Consensus</i>		ATG					
<i>Arabidopsis</i>	PSTISTKSSS	PRR..LFF..					433
<i>Anabaena</i>	PKEKS.....	GLDI	PDFLQRRRPP	KN.....			428
<i>B. subtilis</i>	QMTVSRRSTP	PAD..DTLDI	PTFLNRNRKR	G.....			382
<i>S. aureus</i>	TVSVSRTHT	TKE..D..DI	PSFIRNRER	RSRRTR			390
<i>E. coli</i>	AKVVNDNAPQ	TAKEPDYLDI	PAFLRKQAD.				383
<i>R. meliloti</i>							345

FIG. 1 Alignment of *Arabidopsis* FtsZ deduced amino-acid sequence with prokaryotic FtsZ proteins. 'Consensus' indicates invariant residues. Residues common to both FtsZ and tubulins⁶ are indicated by dots, and the open circle identifies a residue identical in tubulins and all FtsZ proteins except *Arabidopsis* FtsZ. The 'tubulin signature' motif⁶ is indicated by a line. The alignment was performed by the Wisconsin Sequence Analysis Package Pileup program. Accession numbers for sequences shown are: *A. thaliana*, T22504; *Anabaena* sp., Z31371; *Bacillus subtilis*, P17865; *Staphylococcus aureus*, U06462; *Rhizobium meliloti*, L25440; and *E. coli*, P06138 (corrected as in ref. 9).

gene from the cyanobacterial genus *Anabaena* (53% identity and 68% similarity; Fig. 1), suggesting that it may be a chloroplast protein of endosymbiotic origin. The amino-terminal 45–55 residues of *Arabidopsis* FtsZ have properties typical of chloroplast transit peptides, including a high proportion of hydroxylated amino acids, paucity of acidic residues, and alanine as the second residue⁷ (Fig. 1). An *in vitro* chloroplast import experiment (Fig. 2) revealed that the full-length *Arabidopsis* FtsZ translation product (lane 1)

can be imported posttranslationally into chloroplasts where the putative transit peptide is cleaved (lane 2), yielding a soluble, processed form of the protein which is protected from proteinase treatment (lane 3) unless the chloroplast membranes are disrupted with detergent (lane 4). The *Arabidopsis* FtsZ homologue thus seems to be a nuclear-encoded protein localized in the stromal compartment of the chloroplast.

The high degree of similarity between bacterial FtsZ and this chloroplast homologue (cpFtsZ) suggests that cpFtsZ may

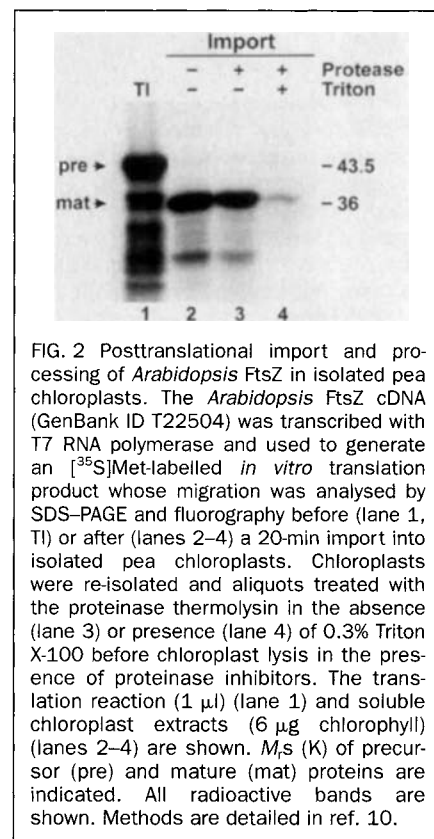


FIG. 2 Posttranslational import and processing of *Arabidopsis* FtsZ in isolated pea chloroplasts. The *Arabidopsis* FtsZ cDNA (GenBank ID T22504) was transcribed with T7 RNA polymerase and used to generate an [³⁵S]Met-labelled *in vitro* translation product whose migration was analysed by SDS-PAGE and fluorography before (lane 1, TI) or after (lanes 2–4) a 20-min import into isolated pea chloroplasts. Chloroplasts were re-isolated and aliquots treated with the proteinase thermolysin in the absence (lane 3) or presence (lane 4) of 0.3% Triton X-100 before chloroplast lysis in the presence of proteinase inhibitors. The translation reaction (1 μ l) (lane 1) and soluble chloroplast extracts (6 μ g chlorophyll) (lanes 2–4) are shown. M_s (K) of precursor (pre) and mature (mat) proteins are indicated. All radioactive bands are shown. Methods are detailed in ref. 10.

be a component of the chloroplast division machinery in plant cells. Several studies have documented the presence of an electron-dense ring at the division site in chloroplasts⁸ resembling the FtsZ ring in dividing bacteria. Identification of the *cpFtsZ* gene provides molecular evidence for the conservation of division mechanisms during the evolution of plastids from their endosymbiotic progenitors, and further establishes a precedent for the existence of a cytoskeletal-like protein in a eukaryotic organelle. It is reasonable to suppose that a mitochondrial FtsZ homologue and possibly organellar homologues of other bacterial division genes will be identified. Meanwhile, the *cpFtsZ* gene provides a starting point for investigating the mechanisms of chloroplast division and the underlying regulation of chloroplast number in different cell types and under different environmental conditions.

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Scorpions in a bottle

Lorna Arnold

Dark Sun: The Making of the Hydrogen Bomb. By Richard Rhodes. *Simon and Schuster*: 1995. Pp. 731. \$32.50.

RICHARD Rhodes weaves many complex layers of material into a large book with a powerful unifying theme: how the invention of nuclear and thermonuclear weapons in this century "introduced a new singularity into the human world — a deep new reality, a region where the old rules of war no longer applied", where the word "omnicide" acquired meaning.

The story of this awesome invention is

Cuban missile crisis. The huge cast list includes the anonymous zeks — political prisoners in the terrible labour camps that built the Soviet atomic installations. Famous (and other) characters are vividly presented — US Presidents Harry S. Truman, Dwight D. Eisenhower and John F. Kennedy, Lavrenti Beria, General Curtis LeMay, Robert Oppenheimer, Hans Bethe, Edward Teller, Klaus Fuchs, Igor Kurchatov, Yuli Khari-

lis, Marshall Rosenbluth, Robert Serber, Edward Teller and Herbert York and the Soviet physicists Lev Altschuler and Yuli Khariton.

Who invented the hydrogen bomb? Norris Bradbury, first director of postwar Los Alamos, said: "Don't ask me who the father of the H bomb was, because nobody is. . . . It wasn't anybody's real invention; it was just a lot of people working on it." Rhodes gives the fullest and most illuminating account I have seen of the famous collaboration between Edward Teller and Stanislaw Ulam and of how Teller's "classical super" was finally shown to be unworkable and was replaced by the new ideas that bear their names. It is clearer than ever that the US hydrogen bomb was truly "the work of many people", although Teller seems to have found it intolerable that someone might share the credit for the historic invention on which he worked single-mindedly for almost ten years (but which, it is argued, he perhaps delayed).

Hans Bethe, in a nearly contemporary review, had said that the discovery was "about as surprising as the discovery of fission had been to physicists in 1939. . . . Such miracles do happen occasionally in scientific history." But Niels Bohr thought that if one had asked a good class of physics students, two or three of them would have suggested the solution. Carson Mark, connecting the invention with the experimental George shot in the 1951 Greenhouse test series, commented on the idea of radiation implosion:

It's true that the Teller-Ulam scheme involved radiation. It's true that Ulam thought that you could use. . . hydrodynamic shock [to implode the secondary; the staged bomb was Ulam's idea]. That's certainly there too. It doesn't move as rapidly as radiation does. . . . So if you were trying to exercise Ulam's idea of hydrodynamic shock you'd find, by God, radiation gets there first. Teller then is supposed to have proposed that it would be better to use radiation. . . . Well, he was right, it was simpler, but you couldn't have avoided it. Had you sat down to design the thing. . . you'd say, dear God, the radiation is going faster. . . so let's concentrate on that. . . . The fact that Teller thought of radiation was natural because he had been involved in much more detailed work on the George shot than had Ulam.

The Soviet development of thermonuclear ideas by Andrei Sakharov (in his "layer cake" design) and then Vitaly Ginzburg (in the use of lithium deuteride instead of deuterium and tritium in the fusion layer), before they went on to staged designs, is interestingly described though in less detail. Rhodes had substantially finished drafting his Soviet narrative when David Holloway's magisterial *Stalin and the Bomb* appeared (Yale University Press; for a review by Hans Bethe, see



The explosion of the Ivy Mike hydrogen bomb superimposed on the Manhattan skyline. Comparison with a similar picture of the explosion of Bikini Baker, a Nagasaki-scale atomic bomb, dramatizes the thousand-fold difference in their destructiveness (see over).

set in a broad context of world politics and war. Rhodes deals with international relations and national policies; military strategy and operations; questions of morality; espionage (an engrossing story); personalities and personal relationships; science and technology. The perspectives are vast but the processes whereby scientific research and abstract concepts were translated into solid numbers and measurements, and then into hardware, are described in careful and precise detail, while the living, breathing human beings are always clearly in view.

The narrative moves between Washington, Moscow, London, Los Alamos, Copenhagen, Chelyabinsk, Eniwetok and Semipalatinsk. It embraces the Berlin blockade, the Korean War (when in three years a fifth of the population was 'killed off' without nuclear assistance) and the

ton and Andrei Sakharov. There is a moving account of the last day in the life of Kurchatov ("the Beard"), waltzing with Khariton's wife to a gramophone record in the kitchen, dying a little later as he sat on a park bench in the winter sunshine, quietly conversing with his old friend Khariton.

The amount of scientific and technical information on thermonuclear devices, all obtained from unclassified and declassified sources, may surprise readers. Rhodes has read widely and has benefited from access to Chuck Hansen's papers, "the best extant collection in private hands of declassified documents relating to weapon design, development and testing". He has also gained much from correspondence and interviews with, for example, the US scientists Hans Bethe, Carson Mark, Nicholas Metropo-

Nature 372, 281; 1994), but he was able to see a proof copy. Their accounts "basically match up".

Rhodes's chapter on the design, construction and testing of the US Ivy Mike, the first ever thermonuclear device, in November 1952 is full of fascinating detail. A huge scientific experiment rather than a weapon test—although the device was later weaponized—it was a cryogenic design using liquid deuterium and a small quantity of tritium. It yielded 10.4 megatons, vaporized the coral isle of Elugelab and produced a cloud 100 miles wide. Its fireball created "every element ever assembled in the universe", plus new transuranic species such as element 100 (later called fermium).

The next US hydrogen bomb trials—the Castle series in the spring of 1954—tested six different designs of staged bombs using solid fuel in the form of lithium deuteride. The first shot, the notorious Bravo, with a greater than expected yield of 15 megatons, contaminated a Japanese fishing vessel, the *Lucky*

Dragon, caused severe fallout on the Marshall Islands and aroused worldwide protest.

To encompass so much in one book is a formidable undertaking, brilliantly carried out and fully justified by the symbiotic Cold War relationship of the two superpowers. Their weapons programmes influenced each other profoundly. It was the first Soviet atom bomb test in August 1949 that panicked the United States into an all-out concentrated effort to develop hydrogen bombs; the extreme vulnerability of the Soviet Union to nuclear attack gave overwhelming urgency to the efforts of the Soviets, for they must have known that some Americans wanted a preventive war and that General LeMay envisaged the destruction of 70 Soviet cities in 30 days, with 6.7 million deaths and casualties among the civilian population.

Espionage was a very important element in this relationship. The Soviet Union obtained many thousands of classified documents, some of which Igor Kurchatov himself considered to be "of

immense value to our science and our country". The espionage factor also greatly influenced US politics.

The final chapter of *Dark Sun* asks some interesting questions: for example, what is a hydrogen bomb and what part have semantics played in US weapon development? (A great deal, apparently, unlike the more rationally conceived Soviet programme). The chapter also asks some profound questions about the past and the future and the 'new singularity'. This most rewarding book is unique in the grim grandeur of its scope, the richness and originality of its content, and its deep and humane understanding. □

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■ *Dark Sun* is also available on four audio-cassettes, read by the author (Simon and Schuster Audio, \$25); and Richard Rhodes's award-winning *The Making of the Atomic Bomb* has just been reissued in paperback by Touchstone Books at \$16.

Remembering the dawn of the nuclear age

SEVERAL other new books have been published to mark the fiftieth anniversary of the atomic bombings of Hiroshima and Nagasaki, the end of the Second World War and the start of the nuclear age.

Concentrating on why the bomb was dropped are **Hiroshima: Why America Dropped the Bomb** (Little, Brown, \$19.95) by Ronald Takaki, who examines Harry Truman's "troubled ambivalence" over the attack, and **The Decision to Use the Bomb** (Knopf, \$32.50) by Gar Alperovitz, who argues that the United States dropped the bomb to impress the Soviets rather than avoid costly invasion.

Such historical controversies about the use of the bomb are neatly summed up by Robert Jay Lifton and Greg Mitchell in **Hiroshima in America: Fifty Years of Denial** (Grosset/Putnam, \$27.50). But their main aim is to show that Americans are still unwilling to face the truth about Hiroshima. "By confronting the legacy of Hiroshima," they write, "and exploring what it has done to living and dying in the late twentieth century, we embark on a path of psychological and political renewal".

More straightforward factual views appear in Adrian Weale's informative **Eye-Witness Hiroshima** (Robinson/Carrroll and Graf, £5.99/\$10.95), a paper-



The Bikini Baker atomic bomb (see page 475).

back collection of first-hand accounts of the development and use of the atomic bomb, from the perspective of the military, scientists, politicians and survivors.

A meticulously detailed record of the war's last month is given by Stanley Weintraub in **The Last Great Victory** (Dutton, \$35), which interweaves the

momentous events with fascinating personal stories.

And the development of the atom bomb is traced through 280 pictures (many never before published) in **Picturing the Bomb: Photographs from the Secret World of the Manhattan Project** (Abrams, £39.95, September) by Rachel Fermi (granddaughter of Enrico Fermi) and Esther Samra.

Survivors and witnesses revisit the Japanese bombing in **Nagasaki Journey: The Photographs of Yosuke Yamahata, August 10, 1945** (Pomegranate Artbooks, \$22.95); **Children of the Atomic Bomb: An American Physician's Memoir of Nagasaki, Hiroshima and the Marshall Islands** (Duke University Press, \$16.95) by James N. Yamazaki (with Louis B. Fleming); **Hiroshima Notes** (Marion Boyars, \$22.95) a collection of essays from the 1960s by the Japanese pacifist and novelist Kenzaburo Oe, winner of last year's

Nobel prize for literature; **White Flash, Black Rain: Women of Japan Relive the Bomb** (Milkweed Editions, \$12.95), an anthology of prose and poetry; and **Writing Ground Zero: Japanese Literature and the Atomic Bomb** by John Whittier Treat (University of Chicago Press, \$34.95).

Peter Tallack

The science of politics

Jacqueline Reynolds

Science and the Founding Fathers. By I. Bernard Cohen. Norton: 1995. Pp. 368. \$25, £17.95.

THE hallmark of the eighteenth century, *le siècle des lumières*, was revolution — revolution of human thought as well as actual insurrection against established governments. This was the age in which old dogma passed away, reason was held to be supreme, science flourished in the salon as well as the laboratory. Newtonian principles and Lockian philosophy were as much a part of the scene as were the fine arts. At the same time and amid much bloodshed, France brutally deposed a hated monarchy, and British subjects in the New World ousted the troops of George III, founding a new republic. Among the principal players in these political and martial events were renowned intellectuals of the day.

In *Science and the Founding Fathers*, I. Bernard Cohen examines the role that science played in shaping the political thought of some of these eighteenth-century Americans — principally, Thomas Jefferson, Benjamin Franklin, John Adams and James Madison. Not surprisingly, we find much of their writing and political rhetoric sprinkled with scientific analogies and metaphors. References to science, whether direct or indirect, gave an authoritative ring to political argument and appealed to the popular desire to place all areas of human endeavour in the realm of reason and rationality. Perhaps of more interest is the fact that the repeated use of scientific metaphor by politicians implies a familiarity on the part of the audience with the details and nuances of the science of the day, a situation very different from the near scientific illiteracy of our own age. Can one imagine a modern prime minister or president using Newton's *Principia* as a basis for political analogy? For that matter, can one imagine a modern prime minister or president even reading the *Principia*?

Cohen devotes separate chapters to Jefferson, Franklin and Adams and a final section to "Science and the Constitution". We are introduced to Jefferson as a polymath actively involved in agriculture, mathematics, physics, astronomy, natural history, palaeontology and archaeology. The author takes us through Franklin's

principal contributions to the science of electricity and Adams's somewhat confused ideas about Newtonian mechanics. But the main thrust of the book is to connect these scientific activities with the more public part of their lives, to show "that the history of science can illuminate some of the central aspects of American history".

A few examples should suffice to provide the flavour of Cohen's thesis. Thus Jefferson, the architect of the Declaration of Independence, used the phrase "the laws of nature and nature's God" — words that were common parlance in the

a problem that seems to recur in the writings of some historians and biographers. Cohen is at such pains to correct this misunderstanding of the contents of the *Principia* that he dwells at length on this topic in eight different sections of his book — a slightly irritating and unnecessary repetition.

A further example of the influence of science in the newly formed United States is found in the political and public life of Franklin. His principal role for many years was in representing his country and his country's interests abroad where his influence came not only from the force of his personality (common sense and general good will) but also from the esteem in which he was held for his scientific accomplishments.

Finally, we come to consider that most important of political documents, the Constitution of the United States of America, drafted by James Madison and his fellow representatives to the Constitutional Convention. (Note that Jefferson and Adams were not a part of this group, and that Franklin, who was present, argued unsuccessfully for a unicameral legislature.) Some American historians and political scientists have postulated that this document is Newtonian in origin, an interpretation roundly rejected by Cohen and apparently attributable in the first instance to Woodrow Wilson — another of our characters who misunderstood the thrust of Newtonian mechanics.

Science and the Founding Fathers ends with a collection of 12 short essays (in the modern sense) covering an extraordinary range of subjects, for example "The meaning of science and experiment", "Jefferson and the Megalonyx or Megatherium" and "Adams' speculations about respiration and magnetism". These supplements enlarge upon themes elsewhere in the book but could well stand alone, a brief testimony to the interweaving of eighteenth-century science with the fabric of political life.

Cohen is a respected historian, noted especially for his studies of the works of Franklin and Newton, and is well placed to judge the influence of scientific thought on early American political life. Historians and scientists should find this book rewarding, a reminder of the days when science and society marched hand in hand. It might even enlighten a few politicians if they could be persuaded to read it. □

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This print, published in 1760 a year before Jefferson entered the College of William and Mary, Williamsburg, as a freshman, shows the master of natural philosophy demonstrating the features of the orrery.

eighteenth century and held a special scientific significance. Primarily, this was a Cartesian expression that was universally adopted for the fundamental principles of science, and its use reflects both Jefferson's own background and its association in the minds of men of learning with Newtonian axioms.

Similarly, Adams invoked the physical concepts of balance and equilibrium in support of his strong views on the necessity for checks and balances within the structure of government. Cohen comments on Adams's *Defence of the Constitution of Government of the United States of America*: "one is sometimes hard put to discern whether he is writing about politics and social issues or about the science of statics". But Adams did not have the grasp of Newtonian physics of some of his contemporaries and not infrequently confused the principles of mechanical dynamics with those of static equilibrium,

From *Science and the Founding Fathers*

Playing with fire

Stanley N. Williams

Monitoring Active Volcanoes: Strategies, Procedures and Techniques. By Bill McGuire, Christopher Kilburn and John Murray. UCL Press: 1995. Pp. 421. £65, \$99. Distributed in the United States by Taylor and Francis.

MONITORING an active volcano is no mean feat. This is the first book to take on the formidable task of bringing together the strategies, procedures and techniques involved. It seems that the editors, with the help of 25 other authors, have had some success. I doubt whether my graduate students will need to buy the book for the advanced course in monitoring volcanoes that I plan to teach next year, but it should certainly be useful as a general reference.

There are 1,500 active volcanoes scattered around the world, and many useful and important things can be learnt from each of them. Those who really need a good textbook on methodology are in the developing countries, where some 500 million people are at risk from active volcanoes. Although there are many international leaders in monitoring active volcanoes, the contributors here are essentially all British, Italian and French; the lack of Japanese, Soviet, Icelandic and US authors, for example, means that the book has a rather limited perspective, focusing as it does mainly on Mount Etna (Sicily) and Piton de la Fournaise (Réunion). It is of course good that we can concentrate on dependably active volcanoes, but to do so at the expense of others misses many of the big differences in the goals of interesting basic research and the way in which active volcanoes are monitored worldwide. Seismology is the established and best method of monitoring volcanoes but the one chapter on that subject is very limited in its approach, ignores methodology and mentions nothing of the need for portable systems to deal with crises that crop up. The chapter on forecasting the behaviour of lava flows outlines an approach that in fact has little monitoring value, even though monitoring is the subject of the book.

John Murray and others do a great job of discussing how their approach to deformation can be made useful. Particularly enjoyable is the page devoted to vandalism and the authors' clever way of dealing with people and their bedevilment — a truly international problem. The chapter goes on to describe a suitably informative combination of methodology, instrumentation and results. Hazel Rymer contributes a similarly wonderful chapter on microgravity monitoring, showing how to get numbers from the field instruments.

The reality of monitoring active volca-

noes is that we need many improvements in instrumentation and methodology. Despite the fact that the authors do not seriously address current problems and possible solutions, I am glad to have the book available, although it would be more valuable if the level of the chapters were not so unequal. Having said this, my recent time spent monitoring active volcanoes in Latin America, Indonesia and elsewhere may have made me difficult to please. □

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Fossil fish

Keith Stewart Thomson

The Rise of Fishes: 500 Million Years of Evolution. By John A. Long. Johns Hopkins University Press: 1995. Pp. 223. \$49.95.

IT is an odd fact that despite the great importance of fossil fishes, especially from the Palaeozoic and Early Mesozoic, in the development of both stratigraphy and evolutionary biology, very few books have been devoted to them. The first of course was Louis Agassiz's *Poissons Fossiles* (1833–44). After that, apart from more technical treatises, we have only had J. A. Moy-Thomas's superb little monograph *Palaeozoic Fishes* (1939), followed by Roger Miles's excellent revision and amplification (1971). Now comes Long's *tour de force*.

The Rise of Fishes is a mixture of text almost as detailed as the two versions of Moy-Thomas's book together with a great deal of background information and, above all, hundreds of superb

photographs and drawings. One could almost take this for a coffee-table book at first glance, so profusely and well is it illustrated. Most of the photographs are by the author himself, who has been diligent beyond any reasonable expectation in studying at first hand the full range of materials about which he has written. Each group of fishes and almost all the important genera are discussed, with special emphasis on the newer taxa from the southern continents.

One great development in the past 30 years of fish evolution has been the explosion of discoveries from Australia, South America and Antarctica. The fishes of Gondwana reveal a range of diversity unknown elsewhere, including many early taxa arguably of crucial evolutionary significance. If Long allows himself a little Australo-centrism, the richness of anti-podean material with which he and workers such as Alex Ritchie (Australian Museum) have dazzled us northerners justifies it. Added to discoveries in South America by John Maisey and others and the extraordinary finds in China and South-East Asia, they force us to rewrite our vertebrate histories, which have been centred on Europe, indeed on Agassiz's Old Red Sandstone, for far too long.

This book is not just a scientific and educational tool; it is also a visual celebration of the glories of biological diversity as seen in the fossil record. It will come as a surprise to many who have not realized how far the palaeontology of early vertebrates has progressed or experienced the depth of evolutionary information available from fossils. And, best of all, there is clearly more to come. □

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Eastmanosteus, a Late Devonian placoderm from east Gogo Station, Western Australia.

From *The Rise of Fishes*

Homeotic genes and the evolution of arthropods and chordates

Sean B. Carroll

Clusters of homeotic genes sculpt the morphology of animal body plans and body parts. Different body patterns may evolve through changes in homeotic gene number, regulation or function. Recent evidence suggests that homeotic gene clusters were duplicated early in vertebrate evolution, but the generation of arthropod and tetrapod diversity has largely involved regulatory changes in the expression of conserved arrays of homeotic genes and the evolution of interactions between homeotic proteins and the genes they regulate.

ARTHROPODS, in terms of their diversity and sheer numbers, and chordates, because of their anatomical and behavioural complexity, are two of the most successful animal phyla. The evolution of both groups has been marked by numerous innovations and modifications to their respective body plans, the archetypes of which arose more than 500 million years ago¹. Although arthropod and vertebrate phylogeny have long been approached through systematics and palaeontology, the genetic basis for the morphological diversity of these or any other animals has, until recently, been beyond the reach of biology.

How do body plans and body parts evolve? Differences in morphology are, of course, the developmental products of genetic differences between animals, but it has not been clear how many or what kind of differences underlie changes in body patterns. Do gross changes in morphology, such as the evolution of fish, snakes and mammals, or the invention of the insect wing, involve distinct genetic mechanisms from the evolution of different kinds of butterflies?

Early investigations into the nature of genetic evolution identified two potential mechanisms underlying the origin of new features. Ohno², for example, stressed the role of gene duplication and divergence in evolution, whereas Wilson³ and Jacob⁴ emphasized the power of regulatory changes in gene expression. But in order to determine how new genes or regulatory innovations might affect morphological evolution, it is essential to know which genes control morphology^{3,5,6}.

One of the most important biological discoveries of the past decade is that arthropods and chordates, and indeed most or all other animals, share a special family of genes, the homeotic (or *Hox*) genes, which are important for determining body pattern. The diversity of *Hox*-regulated features in arthropods (segment morphology, appendage number and pattern) and vertebrates (vertebral morphology, limb and central nervous system pattern) suggests that the *Hox* genes are implicated at some level in the morphological evolution of these animals.

After a brief review of *Hox* gene organization and function in development, I will describe several examples of morphological differences among arthropods and chordates involving evolution at the *Hox* gene level that have recently been discovered. These include changes in the regulation of *Hox* genes and the evolution of interactions between *Hox* gene products and the genes they regulate. The evidence indicates that the duplication of *Hox* clusters and other developmental genes between primitive chordates and early vertebrates enabled the evolution of the anatomical complexity of vertebrates. However, the diversity of arthropods, insects and vertebrates has arisen primarily through regulatory evolution.

Homeotic genes in development

Early studies of homeotic genes were almost entirely restricted to *Drosophila melanogaster*, where these genes were known to

control the identity (that is, the unique appearance) of different body segments along the anteroposterior (A-P) axis of the embryo and adult fly. Subsequently, *Hox* genes have been found in all sorts of animals, including hydra⁷, nematodes⁸, and all arthropods⁹ and chordates¹⁰. Three remarkable conserved features unite the *Hox* genes of higher animals: (1) their organization in gene complexes; (2) their expression in discrete regions in the same relative order along the main (A-P) body axis¹¹; and (3) their possession of a sequence of 180 base pairs (the homeobox) encoding a DNA-binding motif (the homeodomain).

***Hox* gene organization and expression.** In *Drosophila* there are eight *Hox* genes among two complexes, the Antennapedia Complex (ANT-C)¹² and Bithorax Complex (BX-C)¹³, which act in different regions of the animal (other homeobox genes of the ANT-C that are not homeotic genes will not be considered here; see ref. 9 for discussion). There is a striking correlation between the order of these genes on the chromosome and the position of their expression in the developing animal (Fig. 1, top). In beetles, one gene complex contains the same set of genes, and they control the unique appearance of different beetle segments in an anterior to posterior order¹⁴. Vertebrate *Hox* genes are also organized in clusters which are deployed in an anterior to posterior order according to their position in the complex¹⁵. Vertebrates have four clusters of 9 to 11 *Hox* genes which can be aligned into 13 sets (called paralogous groups) based upon their sequence, organization and thus homology to their *Drosophila* relatives (Fig. 1). Not all genes are represented in each vertebrate cluster, and some genes within clusters appear to have been duplicated since the invertebrate/vertebrate divergence. For example, the *Hox* gene most similar to *Abdominal-B* has been duplicated several times and exists in multiple copies (*Hox-9-13*) in three of the four clusters (A, C and D), suggesting that its representation was expanded before the clusters were duplicated.

The conservation of *Hox* genes may at first seem paradoxical. How can animals that have the same array of *Hox* genes appear so different? For example, flies and beetles have the same complement of *Hox* genes. Moreover, mammals and teleost fish¹⁶ (R. Krumlauf, personal communication; D. Duboule, personal communication) each have four *Hox* clusters (and at least the *Hox B* clusters contain the same set of genes (R. Krumlauf, personal communication)), and these genes are expressed in a conserved relative order along the main body axis¹¹. For insight into this puzzle, we must appreciate that *Hox* genes act only to demarcate relative positions in animals rather than to specify any particular structure¹¹. Within one species, different *Hox* genes control the morphology of different body regions (without *Hox* genes all insect segments appear alike^{13,17}). Between species, the same *Hox* gene can regulate the homologous segment or body region in different ways. The key to understanding how *Hox*

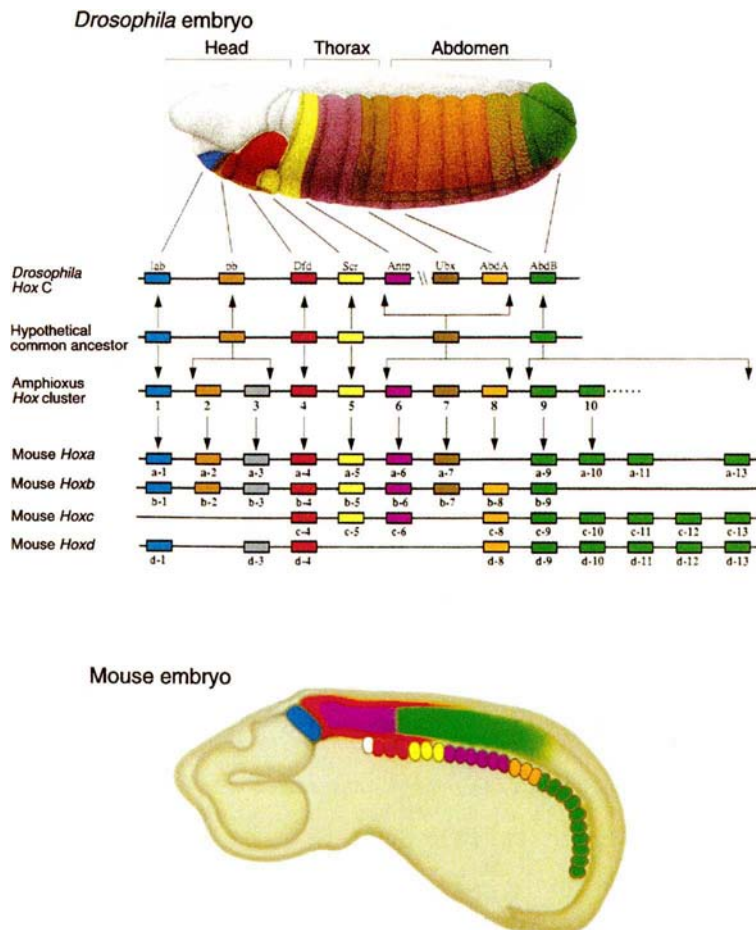


FIG. 1 *Hox* gene organization and expression. Top, the A-P domains of *Drosophila Hox* gene expression correspond to the order of the genes within the *Hox* complex. Middle, the evolutionary relationship between the *Drosophila*, *Amphioxus* and mouse *Hox* clusters, and the deduced complement of *Hox* genes in the presumed common ancestor of arthropods and chordates. Bottom, the A-P domains of mouse *Hox* genes within the developing mouse also correspond to gene order in the *Hox* complexes. Adapted from refs 50, 52 and 75.

genes control morphology and diversity is based on their action as regulatory proteins and the wide range of target genes regulated by different *Hox* genes in one animal, and by the same *Hox* gene in different animals.

Hox proteins and the genes they regulate. The large effects of *Hox* genes on morphology suggest that they regulate, directly and indirectly, large numbers of genes. The promiscuity of *Hox* protein function may stem from the relatively simple sequences recognized by this class of DNA-binding proteins. The *Hox* proteins bind to a four-base core sequence^{18,19}, and there is a lot of flexibility concerning the sequence surrounding this core. This makes it easy to imagine how *Hox* binding sites can be gained or lost by target genes. Indeed, the regulation of several *in vivo* targets of the yeast $\alpha 2$ homeodomain protein has been examined²⁰, and a notable tolerance for different bases within $\alpha 2$

recognition sites was found. Most mutations in target sequences produced only small effects on homeodomain binding *in vitro* and target expression *in vivo*, suggesting that the 'relaxed' DNA-binding specificity of homeodomains may allow new regulatory interactions to evolve readily among *Hox* proteins and potential target genes.

The identity of genes regulated by the *Hox* proteins is a crucial issue because these genes must mediate the cellular processes involved in morphogenesis. In *Drosophila*, proven *Hox* targets include genes encoding other transcriptional regulatory proteins, secreted signalling proteins, and structural proteins, but these few examples may be just the tip of the iceberg²¹. It has recently been estimated that the *Drosophila* genome contains 85 to 170 genes that are regulated by the product of the *Ultrabithorax* gene²². This estimation underscores the monumental challenge

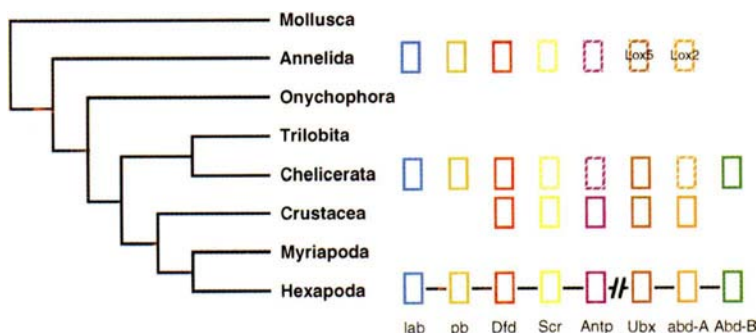


FIG. 2 *Hox* genes and arthropod phylogeny. The array of *Hox* genes detected in arthropods and other invertebrates is displayed in comparison to the *Drosophila Hox* genes. By comparing annelids with arthropods and insects, it appears that most *Hox* genes pre-date the annelid/arthropod divergence, and all insect *Hox* genes pre-date the insect/crustacean divergence. Solid boxes represent confirmed *Hox* genes; stippled boxes are *Hox* genes that are too similar to another to be distinguished on the basis of available data; missing boxes represent the absence of information. Data are from surveys of the *Hox* genes of annelids²⁹, chelicerates⁵⁹ and crustacea²⁸; the arthropod phylogeny on the left is from ref. 76. Other arthropod phylogenies are possible but do not affect the conclusions regarding *Hox* gene origins.

ahead in deciphering how *Ubx* regulation of these genes in *Drosophila* distinguishes the morphology of body segments and appendages. It also helps to explain why the homeodomain protein sequences are so constrained in evolution (because mutations in the *Ubx* protein would alter the regulation, directly and indirectly, of potentially more than 100 genes). Finally, it suggests that divergence among these large sets of target genes may distinguish animal patterns.

Homeotic genes in evolution

There are six potential genetic mechanisms through which *Hox* genes could influence morphological evolution: (1) an expansion in the structural diversity of *Hox* genes within a *Hox* complex (for example, when did the different *Hox* genes such as *lab*, *pb*, *Dfd*, *Antp* and *abd-B* arise?); (2) an expansion in the number of *Hox* genes of a given class (for example, the multiple *abd-B*-like genes (paralogues 9–13) found in vertebrates); (3) an expansion in the number of *Hox* complexes; (4) the loss of one or more *Hox* genes; (5) a change in the position, timing or level of *Hox* gene expression; and (6) changes in the regulatory interactions between *Hox* proteins and their targets. Several of these mechanisms are now correlated with the diversification of arthropods and chordates.

The Lewis hypothesis: new genes for new animals? In his landmark 1978 review, years before any *Hox* genes had been cloned, Lewis proposed one of the first explicit hypotheses concerning the genetic basis of morphological evolution, in this case the evolution of insects and flies¹³. Founded upon his pioneering genetic studies of homeotic genes in *Drosophila*, this 'Lewis hypothesis' consisted of three central ideas. First, during the evolution of insects, 'leg-suppressing genes' evolved which removed legs from abdominal segments of millipede-like ancestors. Second, 'haltere-promoting genes' evolved to suppress the second pair of wings of a four-winged ancestor. Third, a tandem array of redundant genes presumably diversified by mutation to produce the BX-C. It was this third idea that prompted the initial structural comparisons of homeotic genes a decade ago, and led directly to the identification of the homeobox^{23,25}. The discovery of the homeobox-encoded DNA-binding motif in each *Drosophila* homeotic gene tied together both the history and the biochemical function of homeotic genes, and provided the crucial tool for the study of *Hox* genes in the development and evolution of animals.

The first two ideas suggest that genes of the BX-C, which control the morphology of the posterior thorax and abdomen of *Drosophila*, arose in the course of insect and fly evolution. A survey of annelid^{26,27} and arthropod^{9,28,29} *Hox* genes demonstrates that this is not the case. All of the arthropod *Hox* genes evolved before the origin of insects, as demonstrated by the occurrence of these genes in crustacea and chelicerates (Fig. 2), two arthropod classes that pre-date the insects. In addition, the unexpectedly diverse array of *Hox* genes that exists in annelids (Fig. 2) suggests that the diversification of the *Hox* genes must have occurred before the annelid/arthropod divergence. Similarly, the array of vertebrate *Hox* genes demonstrates that 6 or 7 *Hox* genes were present in the common ancestor of all three groups^{30,31} (Fig. 1, middle), although it is not known what that creature would have looked like¹.

Evolution of *Hox* gene regulation and arthropod body plans.

If the array of *Hox* genes is conserved among insects, crustacea and chelicerates, how do we explain the different body plans characteristic of these classes? The major differences between arthropods involves the number, type and organization of body appendages, such as antennae, claws, mouthparts and legs, all of which evolved from a common, ancestral arthropod limb³². In insects the homeotic genes regulate where body appendages may form and the type of appendage found on a particular segment. For example, in *Drosophila*, the products of the BX-C suppress limb formation on the abdomen by repressing the *Distal-less* gene which is required for proximodistal axis

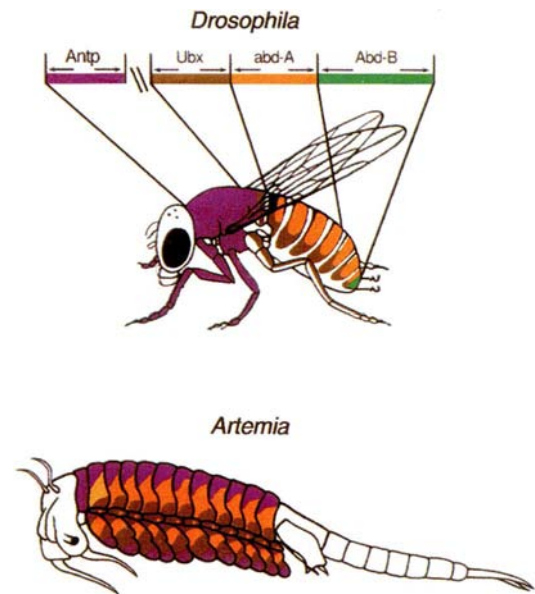


FIG. 3 Evolution of *Hox* gene regulation and the crustacean and insect body plans. The domains of BX-C gene expression have shifted with respect to each other in the thorax and abdomen of insects and crustacea. Top, the *Antp* (purple), *Ubx* (brown), *abd-A* (orange) and *Abd-B* (green) genes sculpt the morphology of the insect trunk. Bottom, *Ubx* and *abd-A* are expressed in relatively more anterior positions in crustacea³⁶.

formation³³, whereas the *Antp* protein promotes leg formation and suppresses antennal development in the second thoracic segment³⁴. The homeotic genes are not actually required to make a limb^{17,35}, as that potential appears to exist in all segments; rather, the homeotic genes either suppress limb development or modify it to create unique appendage morphologies.

The insect body plan is divided into three main body regions (or tagma), with a head bearing numerous appendages, a thorax bearing three pairs of walking legs, and a limbless abdomen. This pattern is sculpted by the homeotic genes. By contrast, chelicerates have just two distinct tagma, and the number and type of appendages vary considerably within the three crustacean tagma³². To determine the extent to which the deployment of homeotic genes reflects the tagmatization of the arthropods, the expression of the three *Hox* proteins, *Antp*, *Ubx* and *abd-A*, has been examined in a crustacean³⁶. Surprisingly, *Ubx* and *abd-A* are expressed in nearly all of the limb-bearing thoracic segments, far more anteriorly than in insects (Fig. 3). This demonstrates that the expression domains of *Hox* genes have shifted considerably between crustacea and insects, and that the interactions between *Hox* proteins and some key developmental programs, such as limb formation, have changed as well.

The scope of regulatory evolution among the *Hox* genes of arthropods is not yet known. The analysis of different crustacea (which exhibit a wide range of body plans³²), chelicerates, myriapods and the putative sister group of the arthropods, the onychophora, will be crucial. It is of special interest to know where the same *Hox* genes are deployed in these last two groups, in which all trunk segments are alike, as this represents the probable ancestral arthropod condition. The *Hox* genes are likely to be present, but they might not regulate limb formation or even ectodermal patterns in these animals.

Modifications of the insect body plan

It now seems likely that all insect diversity has evolved from a body plan sculpted by the same set of homeotic genes. Yet the insect body plan has not been static; there have been numerous changes in the number and type of appendages, which are

reflected by extant and extinct orders. For example, differences in larval limb and adult wing number, as well as appendage morphology, are regulated by homeotic genes. The study of these characters in modern insects may allow us to gain fundamental insights into their origin and diversification.

Prolegs: a *Hox*-regulated atavism? Abdominal limbs known as prolegs are found on the larvae of various insect species belonging to several orders, but are ubiquitous in the Lepidoptera (moths and butterflies). These limbs were probably present in insect ancestors so it may be that prolegs have reappeared through the de-repression of an ancestral limb developmental

program (that is, they may be atavistic). To investigate how homeotic genes influence proleg formation, the expression of several homeotic genes during butterfly development was examined. Proleg formation involves an obvious change in the regulation of *abd-A* expression during embryogenesis³⁷. The initial expression of this gene is in the anterior abdomen, as in all insects examined thus far (where it represses limb formation), but it is selectively turned off in small patches of cells within four abdominal segments which then activate limb-patterning genes and begin to form a proleg. It is not known whether switching off *abd-A* underlies proleg formation in other insects, although the phylogenetic and segmental distribution of prolegs³⁸ suggests that they may arise by a regulatory switch operating on homeotic genes.

The origin and evolution of winged insects.

The most important insect innovation was flight, which catalysed the radiation of the most successful subclass of animals, the pterygotes. What was the role of *Hox* genes in this crucial development? The first pterygote orders are long extinct, so we can only gain insight by studying modern insects. The answer is surprising: *Hox* genes were not involved in the development of wings³⁹. This conclusion relies mainly on the developmental genetics of wing formation in *Drosophila*³⁴, and partly on the available fossil record^{40,41}. In *Drosophila* and all pterygotes, wings form on the second thoracic segment. This is principally the domain of the *Antp* gene, which suggests that *Antp* would be part of a regulatory code for making a wing. However, this is not true because the wing primordia, the imaginal wing disc and the adult wing all appear normal when the *Antp* gene is removed³⁹. Indeed, *Antp* is barely expressed at all in the developing wing^{39,42}. The lack of homeotic input into wing formation makes even more sense when we consider the fossil record. When wings first evolved they appeared on all thoracic and abdominal segments^{40,41}; this lack of segmental restriction suggests that no homeotic gene positively or negatively regulated wing formation.

In the subsequent course of pterygote evolution, however, homeotic genes did indeed become important, but as repressors and modifiers of wing formation. Orders appeared that lacked abdominal wings, and wings were also lost from the first thoracic segment. This suggests homeotic regulation and, in *Drosophila*, the *BX-C* and *Scr* genes do repress the wing primordia in these body segments³⁹. In flies, which bear one pair of wings on the second thoracic segment and a miniature flight appendage (the haltere) on the third thoracic segment, the *Ubx* gene also represses the size of the flight appendage primordia³⁸ and modifies the morphology of the developing haltere⁴³.

The morphological evolution of homologous structures between species. Different *Hox* genes regulate the morphology of serially homologous structures within a species, but what about the homologous structures of different species? In insect terms, for example, can we explain how the hindwings of flies (the haltere), beetles and butterflies are distinct not only from their respective forewings but also from each other? We know in each of these orders that the *Ubx* gene is expressed in and

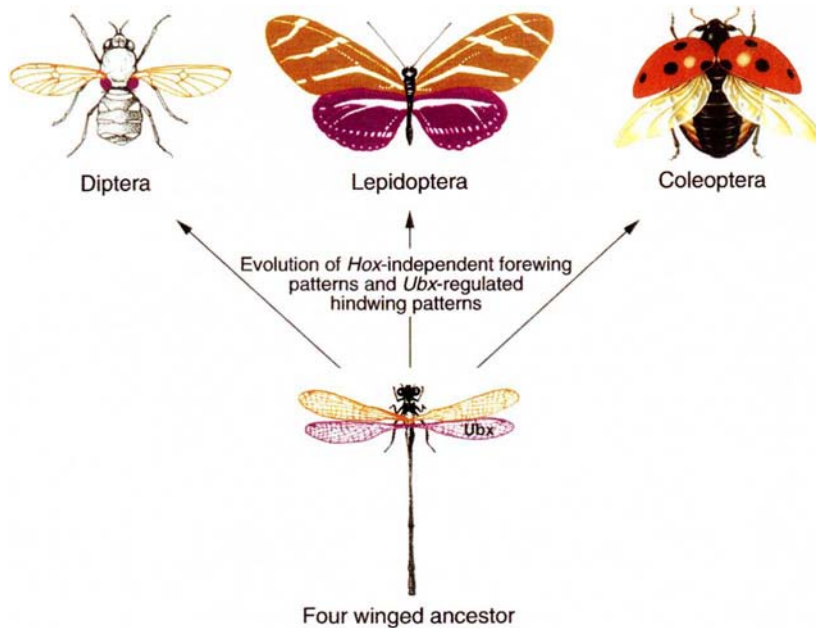


FIG. 4 *Hox* genes and the morphological divergence of homologous structures. The forewings and hindwings differ between insects, and usually from each other. Based on the developmental genetics of wing formation in *Drosophila* and comparisons of homeotic gene expression and function between insects, it appears that forewing patterns evolve independently of homeotic genes, whereas hindwing patterns are *Ubx*-regulated modifications of the respective forewing patterns. In primitive four-winged insects the forewings and hindwings appear similar but have evolved quite different morphologies in various insect orders. The different hindwing morphologies are probably due in part to the divergence of the potentially large sets of wing-patterning genes regulated by *Ubx* in different orders. This divergence is brought about by changes in *cis*-regulatory sequences in target genes that affect their regulation by *Ubx*.

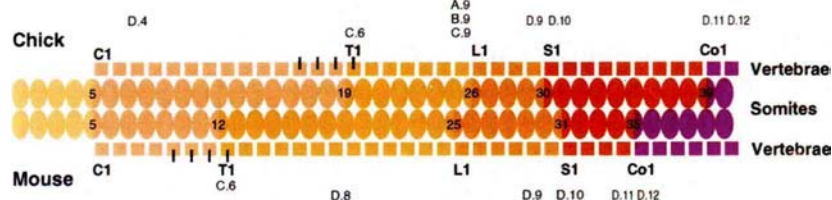


FIG. 5 *Hox* genes and the evolution of vertebrate axial morphology. The expression of various *Hox* genes along the chick and mouse body axes are depicted. The different regions of the respective vertebral columns, that is, cervical (C), thoracic (T), lumbar (L), sacral (S) and caudal (Co), are colour coded. They contain different numbers of vertebrae. The anterior expression boundaries of several members of the *Hox D* cluster mark morphological transitions such as the lumbar/sacral boundary (*Hoxd-9-10*) and the sacral/caudal transition (*Hoxd-11-12*); *Hoxc-6* expression marks the cervical/thoracic transition in both species, even though it arises at different somite positions. Note that in the chick all members of the ninth paralogous group mark the posterior of the thoracic region (*Hoxa-9, b-9, c-9*), except for *Hoxd-9* which is regulated differently and marks the posterior lumbar region. Adapted from ref. 53.

differentiates the hindwing from the forewing^{14,37,44,45}. Because *Antp* is not involved in forewing patterning³⁹, the differences between insect forewings are all independent of homeotic genes. However, the considerable differences in the relative size and morphology of the hindwings must also be due to differences in the wide range of target genes regulated by *Ubx* (Fig. 4). If the set of potential target genes in *Drosophila* is as large as recent studies suggest²², it is easy to imagine that many differences in *Ubx*-regulated target genes exist between species, and that such differences may evolve readily. For example, butterfly hindwing patterns are usually distinct from forewings and are species specific⁴⁶. This diversity could be generated at least in part through the gain or loss of *Ubx* binding sites in the *cis*-regulatory sequences of a myriad of potential target genes.

Rephrasing the Lewis hypothesis. The general theme emerging from developmental studies of *Hox* genes^{10,11,47}, and echoed by the above examples, is that homeotic genes do not constitute an instructional code that says 'make a wing' or 'make a leg'. Rather, they modify developmental programs such that what would otherwise be a wing becomes a haltere, or what would develop as an antenna becomes a leg. In terms of homeotic genes and Lewis's original hypothesis, what has evolved in the course of insect and fly evolution are not new genes but new regulatory interactions between BX-C proteins and genes involved in limb formation and wing morphogenesis.

Hox genes and chordate evolution

The course of vertebrate evolution has been marked by many changes in the chordate body plan. The evolution of the head with paired eyes, jaws and teeth, the origin of the tetrapods and subsequent evolution of limbless forms, and the diverse axial morphologies of modern vertebrates are dramatic examples of large-scale morphological evolution. Developmental analyses of *Hox* gene expression and function implicate the homeotic genes in craniofacial development, hindbrain organization, axial morphology, limb patterning and many other facets of vertebrate development^{15,47}, which begs the question of their potential roles in the evolution of the chordates. The issues parallel those of the arthropods, discussed above: did the appearance of new genes enable the origin of new structures? How have these new structures been modified in the course of evolution?

The examination of these issues in the chordates is facilitated by the relative richness of vertebrate palaeontology, but made more difficult by the genetic and anatomical complexity of the vertebrates. The duplicated *Hox* complexes of modern vertebrates may have allowed the diversification of various homeotic genes but also increases the possibility of genetic redundancy, which conceals individual *Hox* functions in gene knockout experiments⁴⁸. Furthermore, during the formation of a given structure such as a limb, the number of *Hox* genes involved, the spatial and temporal dynamics of gene expression, and the increasing cellular complexity of a growing three-dimensional structure makes individual mutant phenotypes difficult to interpret⁴⁹. Nevertheless, the comparison of *Hox* gene organization and expression in primitive chordates and representative vertebrates has provided several examples of *Hox* gene involvement in chordate evolution.

The origin of the vertebrate body plan. Could the origin of new *Hox* genes underlie the origin of vertebrate characters? To address this question, the organization of *Hox* genes was examined⁵⁰ in amphioxus, a cephalochordate possessing a notochord, dorsal nerve cord, and paired somites, but lacking most vertebrate head structures, appendages and various other features. Remarkably, this primitive creature possesses a single *Hox* complex that appears to be the archetype of *Hox* clusters of modern vertebrates. Two features of the amphioxus cluster stand out. First, it contains homologues of at least the first ten groups of vertebrate *Hox* genes in a collinear array. Second, it contains at least two *Abd-B*-like genes, which indicates that the tandem duplication of these genes and their selective retention

in the cephalochordates preceded, or was at least underway before, the evolution of features unique to vertebrates (such as the limbs in which these genes are important⁴⁹). Thus the origin of new genes via the expansion and diversification of the *Hox* clusters may well have been important in enabling the evolution of more complex chordate body plans.

The number and organization of *Hox* genes in other primitive chordates and vertebrates are now being examined to determine whether there were intermediate stages of *Hox* cluster duplication in the evolution of the vertebrates. In jawless fish (such as lamprey and hagfish) there appear to be fewer *Hox* genes and perhaps as few as two *Hox* clusters (although there could be more)⁵¹. Based upon the available *Hox* data and several other gene families, it has been suggested³⁰ that there were two phases of cluster duplication, one close to the origin of the vertebrates and one close to the origin of jawed vertebrates.

***Hox* gene regulation and the evolution of vertebrate axial morphology.** The mesodermal segments of vertebrates, called somites, are serially homologous and give rise to the vertebrae. The number and morphology of vertebrae that contribute to each distinct region of the vertebral column (the cervical, thoracic, lumbar, sacral and caudal vertebrae) often differs between vertebrates (Fig. 5). For example, mammals (whether mice or giraffes) usually have seven cervical vertebrae, birds have between 13 and 25, and snakes have up to 454 pre-caudal vertebrae. Because *Hox* genes are expressed at distinct levels along the A-P axis of vertebrate embryos, and loss or ectopic expression of individual *Hox* genes can transform vertebrae from one type to another, it has been proposed that the combination of *Hox* genes expressed in each somite specifies the different vertebral morphologies⁵². This would imply that different axial morphologies could be created by shifting *Hox* gene expression domains up or down the A-P axis or by changing the response of *Hox*-regulated genes to a fixed pattern of *Hox* gene expression (for example, at a given somite number).

By comparing the expression of a large set of *Hox* genes between mice and birds, it has been demonstrated⁵³ that *Hox* gene domains shift in parallel with vertebral anatomy and are independent of the number of vertebrae within a given body region (summarized in Fig. 5). For example, *Hox* genes of the fifth paralogue group (for example, *Hoxc-5*) are expressed at the level of the forelimb which arises at somite level 17–18 in the chick and 10–11 in the mouse. Similarly, the anterior expression boundary of *Hoxc-6* falls at the boundary of the neck (cervical) and thorax in mice, chickens, geese (which have 17 cervical vertebrae, 3 more than chickens) and frogs (with a highly derived vertebral morphology and only 3 or 4 cervical vertebrae). The thoracic–lumbar transition appears to be associated with expression of the *Hoxa-9*, *Hoxb-9* and *Hoxc-9* genes, but the fourth member of this paralogue group, *Hoxd-9*, is expressed out of register. This may be significant because the thoracic–lumbar distinction is not general among tetrapods. It may be that shifts within the *Hox-9* group were important in the evolution of this transition from a more uniform trunk, perhaps even in the evolution of the tetrapods from fish. For *Hox* gene expression domains to shift according to anatomy and not somite level, either the upstream regulators of *Hox* gene expression are deployed differently in different vertebrates, and/or the response of *Hox* genes to these regulators has changed. The fact that individual members of paralogous groups have different sites of expression within a species demonstrates that the response to the same upstream regulators can differ, and suggests that the evolution of regulatory diversity within paralogue groups may have enabled major changes in axial morphology (as well as in limb patterns; see below).

Origin of the tetrapods. The earliest tetrapod-like fossils suggest that the vertebrate hindlimb evolved first from the pelvic fin of fish⁵⁴, with the forelimb evolving subsequently from the pectoral fin. It is difficult to explain how the fore- and hindlimbs of tetrapods are so similar to each other when they evolved from

different fish fins. It has been suggested that a major change in *Hox* gene expression brought about the serial homology of the forelimb and hindlimb⁵⁵.

Hox genes are expressed both in the mesoderm adjacent to the limbs and in the developing limb buds of modern tetrapods and are required for anteroposterior and proximodistal patterning. Different *Hox C* genes are expressed in the fore- and hindlimb with their respective expression extending from the adjacent axial mesoderm. However, the *Hox A* and *Hox D* cluster genes expressed in the two limbs are those that are normally expressed in the posterior of the main vertebrate body axis, and they are deployed in very similar spatiotemporal patterns in each limb. Because the *Hox A* and *Hox D* genes are being expressed far anterior to their axial site of expression, it has been suggested⁵⁵ that, in the course of tetrapod evolution, the *Hox A* and *Hox D* cluster genes were ectopically activated in the pectoral fin which led to its transformation from fin to limb and accounts for the serial homology between fore- and hindlimbs of modern terrestrial vertebrates. The ectopic activation of *Hox* genes could most readily be accomplished by the expression of a new signalling centre in the nascent forelimb. The inspection of *Hox* gene expression patterns in living relics, such as the lungfish, coelacanths and sharks, could test the validity of this intriguing model for *Hox* gene function in tetrapod origins.

***Hox* genes and constraint: could snakes learn to walk (again)?** Much of this review has focused on the evolutionary inferences drawn from studies of *Hox* gene organization, expression and function in a few animals. It may also be worthwhile to consider what might be evolutionarily possible but has not been observed (yet). For example, there is no clear case of *Hox* gene loss being implicated in the modification of the vertebrate body plan. Is there such constraint on *Hox* organization and function that even single members of paralogue groups are indispensable? Where might we test such an idea? Perhaps in snakes, which forfeited their limbs, much of their axial morphology, and elements of craniofacial architecture (for example, ears) in the course of their evolution as burrowing reptiles approximately 150 Myr ago⁵⁶. (Their recent radiation as surface-dwelling mammal-hunters is linked to the abundance of prey and the evolution of prey-immobilizing toxins, not the acquisition of new structures.) Given that individual *Hox* genes were lost after the expansion to four *Hox* clusters in vertebrate ancestors⁵⁷, and that mice can survive without certain individual genes¹⁵, it seems possible that snakes could possess fewer *Hox* genes than other vertebrates.

Snakes could reveal what sort of constraints exist on the *Hox* clusters. For example, if the *Hox* genes have all been preserved then regulatory changes can probably explain their unique axial morphology. This could also suggest that the potential for specifying limb position and morphology is still preserved (a possibility supported by the presence of hindlimb rudiments in pythons and boas). If so, is it conceivable that snakes or other limbless tetrapods might regain their limbs? Probably not, as the evolution of limblessness is strongly correlated with body elongation and increased vertebral number, and the constraints against a return towards the primitive tetrapod form may be prohibitive⁵⁸. If *Hox* genes have been lost, however, then we might learn some valuable lessons about genetic redundancy from the identity of the lost genes.

The other side of the constraint issue is illustrated by the horseshoe crab, *Limulus polyphemus*. This chelicerate arthropod may possess up to four *Hox* clusters which include the genes found in the *Drosophila* cluster as well as additional genes present in vertebrate *Hox* clusters⁵⁹. Although the multiple *Hox* clusters could be due to a genome-wide polyploidization event, this must have occurred at least 75 Myr ago. The static body pattern of horseshoe crabs suggests that *Hox* complexity and anatomical complexity are not strictly correlated. If *Hox* genes create possibilities, *Limulus* has apparently not found any creative uses for a lot of *Hox* genes (or other genes), even though

selection has retained them. Might this reflect some constraint imposed by the chelicerate or arthropod body plan that prevents its modification⁶⁰? After 600 Myr to expand their *Hox* clusters, it seems surprising that arthropods don't have more *Hox* genes; is this because they couldn't find ways to use them?

The primacy of regulatory evolution

The available evidence suggests that primitive arthropods and chordates each possessed a single *Hox* complex containing the diverse array of *Hox* genes found in their modern descendants. Although this cluster was duplicated in the chordates, presumably in one or two early phases in their evolution, it appears that the subsequent course of vertebrate evolution from primitive bony fishes to mammals, and the entire course of arthropod evolution, was founded upon conserved sets of *Hox* genes. The phylogeny of *Hox* genes and the many examples cited above of large-scale morphological changes associated with diversity in *Hox* gene regulation and target regulation suggest that the primary genetic mechanism enabling morphological diversity among arthropods and vertebrates is regulatory evolution.

The anatomical complexity of vertebrates, reflected by a larger relative number of different cell types⁶¹, may be a consequence of a greater number of developmental genes. For example, important developmental gene families such as the *Hox*, *Wnt*, *TGF-β* and *Dlx/Dlx* genes are generally several-fold larger in vertebrates than primitive chordates, arthropods or annelids (Table 1 and references cited therein). The structural and regulatory diversity among these genes (especially, for example, the *Wnt* and *TGF-β* families) demonstrates that gene duplication and diversification have probably been an important force in the evolution of the cellular and anatomical complexity of vertebrates. However, the complement of *Hox*^{62,63}, *Wnt*⁶³ and *Dlx*⁶⁴ genes is comparable in fish and mammals. This suggests that morphological diversity within vertebrates is the product of regulatory evolution within larger, but essentially fixed, families of developmental genes.

How can regulatory evolution be sufficient to explain the differences between trilobites and butterflies, or dinosaurs and sparrows? The creative potential of regulatory evolution lies in the hierarchical and combinatorial nature of the regulatory networks that guide the organization of body plans and the morphogenesis of body parts. We now know that *Hox* genes are regulated by many upstream factors, and that *Hox* proteins act as sculptors that modify the basic arthropod or chordate metamer by modulating the expression of potentially dozens of interacting genes, the products of which determine the cellular events of morphogenesis. Variation in the morphogenetic output of such a multigenic network can arise at many levels simply by tinkering with the relative timing of developmental gene expression (heterochrony) or the interactions between members of the regulatory network. Such regulatory changes are presumably the consequence of alterations in *cis*-regulatory sequences through simple mutations as well as duplications or deletions of regulatory elements. In this manner, one aspect of gene function can

TABLE 1 Different trends in chordates and arthropods

	Principal <i>Hox</i> genes	<i>Wnt</i> genes	<i>TGF-β</i> -related genes	<i>Dlx</i> genes
Annelids	≥ 7	≥ 2		
Insects	8	4	3	1
Cephalochordates	≥ 10	≥ 2		
Bony fish	~38	14		≥ 5
Mammals	38	14	>25	6

Gene family data are based on the following references: for *Hox*, refs 15, 27, 50, and D. Duboule and G. Wagner, personal communication; for *Wnt*, refs 30, 63, 70; for *TGF-β*, refs 71, 72; for *Dlx*, refs 64, 73, 74 and, in the zebrafish, M. Westerfield, personal communication.

evolve without altering others. Single genes are often regulated by arrays of discrete regulatory elements which control the pattern, position, timing and level of gene expression, and these features can differ between species⁶⁵. Key body-patterning genes such as the homeotic^{66,67} or *Distal-less*³³ genes contain many such elements, which suggests that the evolution of regulatory elements is much more common, and therefore a more continuous source of variation, than the duplication of entire genes.

Conclusion

Our understanding of the role of *Hox* genes in evolution depends on both developmental and comparative studies. One of the most challenging problems is the elucidation of *Hox* protein function in regulating morphology in model experimental species. When the regulatory targets of individual *Hox* proteins are better known than the differential regulation of these genes by different *Hox* proteins in a single species, or by the same protein in different species, can be assessed. The evolutionary value of this information will rely upon the judicious choice of species selected for comparative study.

At a broader level, it is not known how *Hox* genes are organized or expressed in several major arthropod and vertebrate taxa. At the very least, we need to investigate the *Hox* genes of primitive arthropods and possible sister groups as well as all protochordate and vertebrate classes. Of course, arthropods and chordates are only 2 of the 35 or so metazoan phyla and,

although studying then provides an understanding of general ways of modifying body plans, it does not explain the origins of *Hox* gene diversity or the evolution of other body plans. The exploration of other phyla, such as the cnidaria, echinoderms, molluscs and flatworms, might lead us to discover when this ancient regulatory 'toolbox' came into existence, and to understand its role in the genesis and modification of these diverse animal body plans.

Finally, it must be appreciated that the comparisons of differences between higher taxa only reveal what has changed, not how it changed. We do not know the rate at which these changes arose or the extent of variation in *Hox*-regulated characters in populations. The idea of macroevolution in a single step, the 'hopeful monster' so often insinuated in the discussion of homeotic genes⁶⁸, is widely discredited⁶⁹. The new perspective emerging from the study of *Hox* genes in phylogeny and their regulatory roles in developmental needs to be integrated within the evolutionary frameworks of palaeontology and population biology.

Note added in proof: Sardino *et al.* (*Nature* **375**, 678–681; 1995) have recently shown that the tetrapod foot may be a new structure formed under the control of a deconstruct phase of *Hox* gene expression in the limb bud. □

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The variable effect of clouds on atmospheric absorption of solar radiation

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A four-year global record of solar flux observed from both space and the Earth's surface allows an examination of the effect of clouds on the atmospheric absorption of solar radiation. The results indicate that, contrary to some recent suggestions, the effect of clouds is highly variable and present general circulation models should be able to incorporate cloud absorption into climate simulations.

VARIABLE absorption of solar energy by the Earth's surface and atmosphere is the driving force behind climate and is modulated primarily by clouds. Cloud-radiation interactions are among the most important yet least understood processes controlling climate¹. This seriously hinders general circulation model (GCM) predictions of climate change. A more fundamental concern, however, is that after more than 40 years of work², both theory and observation of the absorption of solar radiation by clouds are still fraught with uncertainties. In essence, cloud absorption inferred from aircraft measurements of solar radiation often exceeds model calculations. Such a discrepancy is referred to as the 'cloud absorption anomaly'. As yet, it is not clear whether this phenomenon is real or an aberration stemming from uncertainties in either aircraft flux observations or input parameters for radiative-transfer modelling³.

Recent studies^{4,6} suggest that the cloud absorption anomaly is not only real but much larger than implied by previous work. These studies employed space-borne, airborne and ground-based observations to infer the effect of clouds on atmospheric absorption of solar radiation by determining the ratio, R , of the short-wavelength cloud radiative forcing (CRF) at the Earth's surface to that at the top of the atmosphere (TOA). They found R to be consistently about 1.5, whereas conventional radiative-transfer models tend to produce R values of about 1.0 (ref. 4). In order for radiative-transfer models to produce $R=1.5$, the specific absorption of cloud droplets would need to be increased uniformly in the near-infrared by a factor of 40 relative to conventional values⁷. If R has a value of 1.5 globally, contemporary radiation models would need to be able to increase their absorption of radiation by cloud particles by $\sim 25 \text{ W m}^{-2}$, on average. In separate studies^{8,9}, it has been demonstrated that such models also underestimate absorption of radiation by clear skies by a similar amount. Taking these findings together implies that the radiation budgets of the atmosphere and surface could differ from those computed by GCMs by up to a factor of two, thus seriously affecting their simulations of the Earth's current climate and their predictions of climate change due to, for example, increasing amounts of greenhouse gases.

Here we determine R following the same methodologies as refs 4 and 5, but using different data sets of longer duration and broader coverage. We find that R is highly variable in the tropics with a median of about 1.4, less variable at mid-latitudes with a median of about 1.1, and consistently less than 1.0 in polar regions. We propose that large values and high variations of R may be related to the presence of absorbing aerosols and the uncertainties in both the observed and inferred solar flux data used here. Therefore, a substantial revision of our understanding of cloud absorption and its impact on the atmosphere's energy budget⁴ may not, after all, prove to be necessary if the effects of absorbing aerosols are properly incorporated.

Determination of cloud effects

The ratio R is defined as

$$R = \frac{\text{CRF}_{\text{SFC}}}{\text{CRF}_{\text{TOA}}} = \frac{F_{\text{SFC}}^{\text{all}} - F_{\text{SFC}}^{\text{clr}}}{F_{\text{TOA}}^{\text{all}} - F_{\text{TOA}}^{\text{clr}}} \quad (1)$$

where CRF_{SFC} and CRF_{TOA} denote CRF at the surface (SFC) and the TOA, respectively. CRF_{SFC} and CRF_{TOA} are defined as differences between net solar radiative fluxes F for all-sky (clear and cloudy skies) and clear-sky conditions indicated by the superscripts. Because it is impossible, in practice, to have simultaneous clear and cloudy measurements, mean clear-sky fluxes were based on available cloudless events over an averaging period. The resulting fluxes are assumed to represent those that would be observed should clear skies occur constantly. This simplification was shown to be a minor problem for monthly-mean solar radiation^{10,11}. As

$$\text{CRF}_{\text{SFC}} = \text{CRF}_{\text{TOA}} - \text{CRF}_{\text{ATM}} \quad (2)$$

where CRF_{ATM} is atmospheric CRF, then

$$\text{CRF}_{\text{ATM}} = (1 - R)\text{CRF}_{\text{TOA}} \quad (3)$$

In general, $\text{CRF}_{\text{TOA}} < 0$ (ref. 12), and so $R > 1$ and $R < 1$ imply, respectively, that clouds enhance and reduce atmospheric absorption relative to clear-sky absorption. The case $R=1$ is when cloud absorption exactly equals the reduction in absorption by gases and aerosols beneath cloud due to reduced transmittance of incoming radiation, less the slight enhancement in absorption by gases and aerosols above cloud due to increased reflectance.

Using the definition in equation (1), Ramanathan *et al.*⁵ obtained R for the western equatorial Pacific Ocean. In their study, all-sky and clear-sky TOA fluxes were taken from satellite measurements. All-sky surface net fluxes were determined as the residual term in the surface heat-balance equation, and clear-sky surface net fluxes were inferred from satellite-based clear-sky TOA fluxes using an inversion algorithm¹³. Cess *et al.*⁴ obtained R from the slope, s , of the linear regression between coincidental TOA albedo and atmospheric transmittance of solar radiation R and s are related by

$$R = -\frac{(1 - \alpha_{\text{SFC}})}{s} \quad (4)$$

where α_{SFC} denotes surface albedo. (Note that the sign of s in equation is opposite to that used in ref. 4.) The advantage of using s instead of R is that there are more observations of surface insolation than surface net solar flux and that estimation of surface albedo is avoided. The disadvantage, however, is that determination of s requires multiple pairs of coincident observations obtained over a period, or area, in order to perform a reliable regression analysis. This will limit the ability to analyse

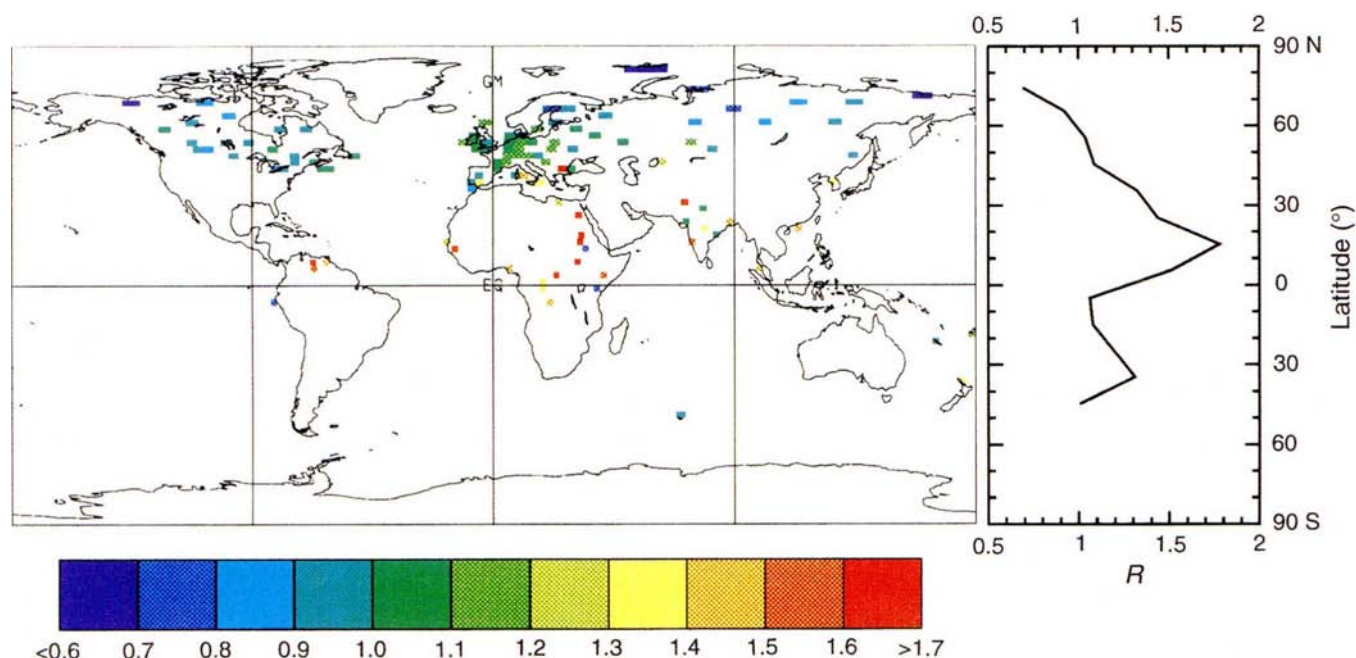


FIG. 1 Left, global distribution of R , the ratio of mean short-wavelength cloud radiative forcing (CRF) at the surface to that at the top of the

atmosphere (TOA) averaged over the entire data period. Right, the corresponding zonal variation of R .

spatial and temporal variations of R . By analysing co-located satellite and surface measurements at various sites, Cess *et al.*⁴ found that values of s are always close to 0.6, whereas GCMs produce values near 0.8. These values of s are approximately equivalent to R of 1.4 and 1.0, respectively⁴.

Both the direct (equation (1)) and the regression methods for determining R were employed in this study. To guide the analyses of the observational results, the sensitivity of R to various factors was examined using a conventional radiative-transfer model¹³. Table 1 lists model-generated diurnal-mean values of R calculated from integration of instantaneous clear and cloudy solar fluxes. Although cloud optical depth was held constant at 40 for the calculations of Table 1, sensitivity tests showed that R is almost insensitive to this variable. The dependence of R on solar zenith angle is shown in ref. 7. R decreases significantly with increasing solar zenith angle, cloud-top altitude and surface albedo. A weaker dependence was found on cloud type that was differentiated here by cloud droplet sizes only¹⁴. It appears from Table 1 that although R depends on a variety of parameters, it rarely exceeds 1.2. These results are for particular profiles of water vapour and aerosols. The results are subject to changes in these profiles. Nevertheless, the maximum diurnal mean value of R never exceeds 1.2 for the combination of typical surface and atmospheric conditions unless there is heavy loading of strongly absorbing aerosol.

Observational data

In our analysis, we used global, monthly-mean measurements from the Earth Radiation Budget Experiment (ERBE)¹⁵ and the Global Energy Balance Archive (GEBA)¹⁶. (ERBE was a three-satellite programme designed to monitor TOA radiative fluxes, while GEBA processed and archived global surface heat-flux measurements.) Gridded monthly-mean ERBE and GEBA data for cells of $280 \times 280 \text{ km}^2$ were extracted from data sets covering the 46 months from March 1985 to December 1988¹⁷. Variable record lengths and the effect of quality control results in number of months per cell varying from fewer than 5 at high latitudes to more than 40 in Germany. Both clear-sky and all-sky TOA

net solar fluxes were obtained from ERBE measurements. Surface all-sky downwelling solar irradiances were observed by pyranometers deployed in the world-wide radiation network. The accuracy of most pyranometers is estimated to be better than 15 W m^{-2} (ref. 16). Surface all-sky albedos and clear-sky net fluxes were inferred from ERBE using the algorithms in refs 18 and 13, respectively. The latter algorithm has been validated for various regions^{5,19}; the former has been shown to give values of clear-sky surface albedo that compare well with an independent algorithm²⁰.

Temporal and spatial differences between satellite and surface measurements give rise to match-up errors. Errors due to time differences are small, as ERBE provides measurements at various local times and the surface data were taken throughout the day. Spatial match-up errors are much reduced by time averaging. The errors that remain depend on the number of surface stations per cell. It has been shown²¹ that match-up errors for monthly data are approximately equal to $24.2/N \text{ (W m}^{-2}\text{)}$ where N is the number of stations per cell; in our study, N ranged from 1 to 10. All matched pairs of satellite and surface data were used except those corresponding to snow-covered land and frozen water which were identified by clear-sky TOA albedos exceeding 0.3. These pairs were excluded for two reasons. First, as seen in Table 1, values of R for bright surfaces differ much from other surfaces. Therefore, the analysis was confined to relatively dark surfaces so that variations in R could be interpreted as changes in atmospheric absorption. Second, ERBE clear-sky identification over snow and ice surfaces is known to be unreliable. After screening, R was computed for each cell on an individual monthly basis; R was also computed for larger time and space domains using the corresponding domain mean values of CRF.

Variability in cloud effects

Figure 1 shows the geographical distribution and latitudinal variation of R computed from mean CRF averaged over the entire data period for every grid. The most striking feature is that R exhibits strong meridional variability. In tropical regions, R is significantly larger than unity and is in apparent agreement with

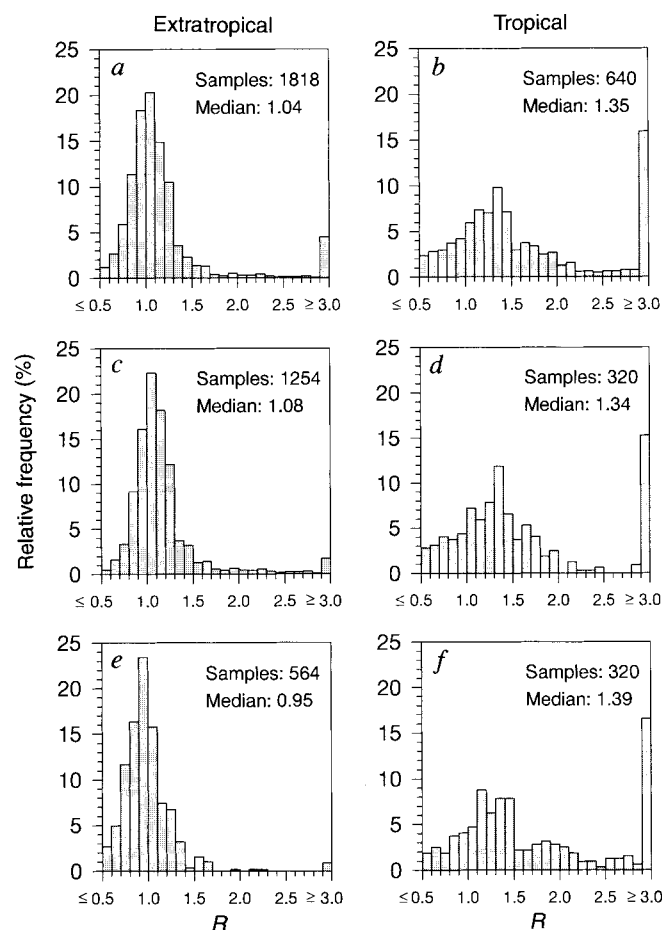


FIG. 2 Relative frequency histograms of monthly R for the tropics (latitude $<30^\circ$) and extratropics (latitude $>30^\circ$) using data from: *a* and *b*, all available months; *c* and *d*, April to September inclusive; *e* and *f*, October to March inclusive. Listed on the plots are number of samples and median values of R .

refs 4–6, implying that tropical clouds enhance atmospheric absorption substantially. For mid-latitudes, R is generally between 1.0 and 1.2, implying that clouds slightly increase total atmospheric absorption. In polar regions, R is less than 1, indicating that polar clouds tend to reduce atmospheric absorption compared to clear-sky absorption. It is likely that these gross variations of R are associated with cloud structure and solar zenith angle. However, it should be pointed out that the tropical GEBA sites are primarily in continental regions close to the sources of strong absorbing aerosols produced by biomass burning^{22–24}, which are, at least, partially responsible for enhanced absorption in the tropics. Unfortunately, lack of such aerosol data on the same time and space scales prevents determining their effects quantitatively. The effect is also manifest in longitudinal variations of R in the mid-latitudes where R is greatest over Europe, intermediate over eastern Canada, and least in central Canada: this pattern corresponds approximately with anthropogenic aerosol loadings²⁵. For western Europe, the largest values of R occur near Hamburg and the Rhine Valley which are known to be heavily polluted regions²⁶. Conversely, cells with the smallest values of R are near southern Spain and Ireland which are relatively non-polluted.

The data were then sub-divided into two regions, tropics (latitudes $\leq 30^\circ$) and extratropics (latitudes $>30^\circ$); they were also divided into three time periods, summer (April–September), winter (October–March) and annual (all months). Figure 2 shows relative frequency histograms of R for individual months and cells for each of the six categories. The most striking feature

of Fig. 2 is that R is even more variable than shown in Fig. 1. On an annual basis, the domain-averaged values of R are 1.38 and 1.06 for tropical and extratropical regions, respectively. The summer and winter histograms in Fig. 2 suggest that R depends on season more in the extratropical regions than in the tropics. A common feature of all the histograms is a sizeable fraction of months with $R < 1$. This may be explained by the abundance of cirriform clouds in many locations²⁷. As these clouds are high, photons are less likely to be absorbed by low-level cloud, aerosol and water vapour (Table 1). Also worth noting is the considerable fraction of cases in the tropics with extremely large values of R (>3). These values of R are barely meaningful, as almost 50% of them occurred when $|\text{CRF}_{\text{TOA}}| < 5 \text{ W m}^{-2}$. Furthermore, small errors in CRF_{TOA} could lead to enormous values of R when $|\text{CRF}_{\text{TOA}}|$ is near zero. Therefore, R in these cases is not significant when assessing the effect of clouds on atmospheric absorption of solar radiation.

Figure 3 shows 94 monthly values of R for three German cells, each of which contained at least seven pyranometers (match-up errors less than 4 W m^{-2}). The seasonality of R is apparent, with smallest and largest values in the winter and summer respectively. Also, the interannual variability of R is striking: variations of 0.2–0.3 are common. The curves in Fig. 3 represent R as simulated by the model used to produce Table 1 for two extreme cloud conditions which favour low and high values of R . Comparing measured R to calculated R suggests that this ratio is much affected by changing distributions of both solar zenith angle (seasonal) and cloud conditions (seasonal and interannual). As the theoretical curves envelop about 85% of the observed values, this may indicate that for mid-latitudes the effect of clouds on total atmospheric absorption is within the reach of conventional radiation models, although discrepancies of smaller magnitude between models and observations are still entirely possible. Considering, however, that the upper envelope in Fig. 3 corresponds to an extreme condition that rarely exists,

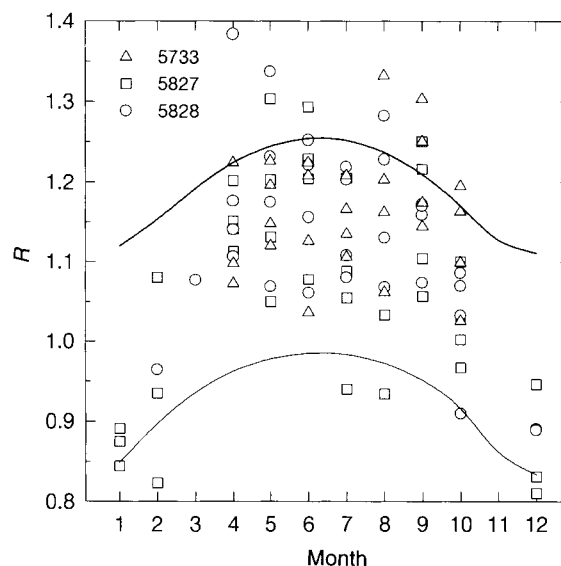


FIG. 3 Intra- and inter-annual variations of R for three German cells denoted by their serial numbers. Latitudes and longitudes at the centres of the cells are $51^\circ 15' \text{ N}$, 2° E for 5827; $51^\circ 15' \text{ N}$, 10° E for 5828; and $48^\circ 45' \text{ N}$, $9^\circ 28' \text{ E}$ for 5733. The curves denote model-simulated diurnal-mean R for conditions favouring extremely low and high values of R . The thick (upper) curve represents a mid-latitude summer atmosphere with continental aerosol of optical depth 0.45 and cumulus cloud¹⁴ of optical depth 40 situated between 0 and 1 km height. The thin (lower) curve corresponds to an aerosol-free, subarctic winter atmosphere with a St-I¹⁴ cloud of optical depth 40 situated between 9 and 13 km height. Land surface at 50° N was used in the calculation of these two curves.

TABLE 1 Sensitivities of R to various factors

Surface	Cloud type	Cloud location (km)	R
Sensitivity to surface type			
Desert	St-I	2-4	0.94
Snow	St-I	2-4	0.64
Water	St-I	2-4	1.03
Crop	St-I	2-4	0.97
Sensitivity to cloud type			
Crop	St-I	2-4	0.97
Crop	Cb	2-4	1.07
Crop	Cu	2-4	1.01
Crop	Sc-II	2-4	1.00
Sensitivity to height of cloud top			
Crop	St-I	0-1	1.17
Crop	St-I	0-2	1.09
Crop	St-I	0-4	1.02
Crop	St-I	0-6	0.98
Crop	St-I	0-9	0.93
Crop	St-I	0-13	0.88
Sensitivity to cloud height			
Crop	St-I	2-4	0.97
Crop	St-I	6-9	0.86

These values of diurnal-mean R were calculated (for 30° N in July) by a doubling-adding code which contained 429 spectral intervals and 8 vertical layers. It accounted for multiple Rayleigh and Mie scattering, and absorption by gases, aerosol and cloud droplets. Henyey-Greenstein phase functions were used. Mid-latitude summer atmosphere³³ conditions, with continental aerosol of optical depth 0.225 at 550 nm wavelength, were employed. Surface broadband albedos for snow, desert, crop and water of 0.91, 0.40, 0.27 and 0.08, respectively, were used. Cloud optical depth was 40 and the size distributions of cloud droplets for four cloud types (stratus (St-I), cumulonimbus (Cb), cumulus (Cu) and stratocumulus (Sc-II)) were as defined in ref. 14.

values of R near and exceeding it represent cases which are beyond these models.

The data used to produce Fig. 3 were also employed to derive R by the regression technique used by Cess *et al.*⁴. These data have the least uncertainties owing to the high density of pyranometers and homogeneous surface types. Figure 4 shows a strong linear relationship between monthly-mean TOA albedo and atmospheric transmittance. The slope of the regression line s is

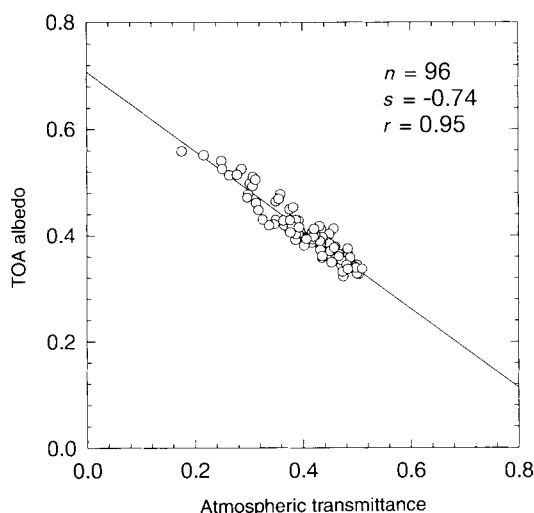


FIG. 4 Scatter plot of monthly-mean TOA albedo measured by ERBE versus monthly-mean atmospheric transmittance determined from surface insolation measurements for the cells used in Fig. 3. n denotes the number of samples, s and r are respectively the slope and correlation coefficient of the linear regression.

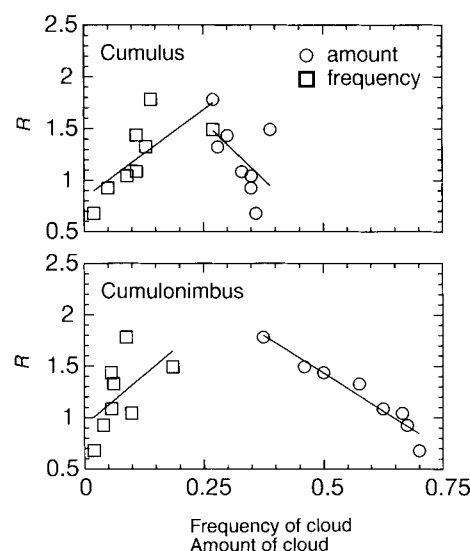


FIG. 5 Annual, zonal-mean values of R in the Northern Hemisphere as a function of corresponding frequency of occurrence and amount (when present) of cumulus (top panel) and cumulonimbus (bottom panel) clouds³¹. Straight lines are least-square, linear-regression fits to the data. Only the slopes with respect to frequency for cumulus, and with respect to amount for cumulonimbus, are significantly different from zero at the 90% confidence level.

-0.74 ± 0.06 (at 95% confidence level) which is slightly less than values generated using GCM data⁴. The mean surface albedo for this region was estimated^{18,20} to be 0.15. Thus the corresponding value of R calculated from equation (4) is 1.15 which coincides well with that derived from the CRFs for the same region. Analyses were also made for 11 individual cells having at least three pyranometers. Slopes were found to vary from -0.67 to -0.87 with a mean of -0.77 with correlation coefficients generally larger than 0.9. The disagreement between our estimate of R for the extratropics, and those of Cess *et al.*⁴ is therefore not due to different analytical methods, but, rather, different data sets (and possibly different averaging periods).

So far, the observed variation of R has been explained by changes in solar zenith angle and aerosol effects. The potential dependence of R on cloud structure also deserves examination. Clouds that most affect the TOA solar radiation budget are cumuliform in the tropics and stratiform in the extratropics²⁸. Thus, it seems reasonable to hypothesize that latitudinal variations in R may be associated partly with differing radiative transfer characteristics stemming from differences in cloud morphology^{29,30}. Figure 5 shows a plot of annual zonal-mean R versus corresponding frequencies of occurrence (fraction of time when clouds are present) and amount (fraction of the sky covered by cloud when they are present) of cumulus (Cu) and cumulonimbus (Cb) clouds³¹. In both cases, R tends to increase as frequency increases and as amount decreases. Hence, it may be tempting to infer from Fig. 5 that enhanced values of R are associated with convective clouds. However, Monte Carlo photon-transport experiments with four spectral bands³² for a variety of towering three-dimensional cloud fields (pure water) in the standard tropical atmosphere³³ yielded diurnal-mean values of R that exceeded their plane-parallel counterpart by no more than 0.15. Moreover, inclusion of reduced single-scattering albedos for large droplets³¹, as characteristic of convective clouds, did not yield significantly larger values of R , but merely enhanced absorption by droplets at the expense of reduced absorption by water vapour in the lower troposphere.

Concluding remarks

In terms of magnitude, estimates of R from this work are not inconsistent with those of recent studies^{4,6} for the tropics, but are at variance with those for other regions⁴. The apparent agree-

ment in the tropics should be treated with scepticism given the potential effects of biomass burning aerosols and large uncertainties in the satellite and ground-based data. Such strongly absorbing aerosols not only increase cloud absorption—and thus the value of R —but also lead to an overestimation of R . This is because the clear-sky surface net solar flux is probably overestimated by the inversion algorithm¹⁵ which only incorporates a small amount of weakly absorbing aerosol. Larger uncertainties of R for the tropics also stem from too-small data samples due to lower pyranometer density and shorter duration of measurements, and too-noisy data associated with the relatively small amounts of thick cloud. In view of these problems, we have much less confidence on the larger values of R found in the tropics than the smaller values of R in the mid-latitudes. Until these problems are resolved, one cannot attribute unambigu-

ously the large values of R to the cloud absorption anomaly. As the discrepancy with ref. 4 for the extratropics is largely related to the use of different data sets, rather than to different analytical methods, it is imperative that the data sets involved undergo detailed intercomparisons. Such an analysis should enable the evaluation of whether a substantial revision of our present understanding of the atmosphere's energy budget resulting from the treatment of cloud absorption as indicated by Cess *et al.* is required.

We note that this study does not rule out the existence of the cloud absorption anomaly, but rather indicates that its magnitude (if it exists) on a global scale may not be as large as suggested in some recent reports. If so, the use of R may not be an effective means of addressing the cloud absorption anomaly. R is not a direct measure of cloud absorption, as its value is influenced by many factors other than clouds. □

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Structure of serum response factor core bound to DNA

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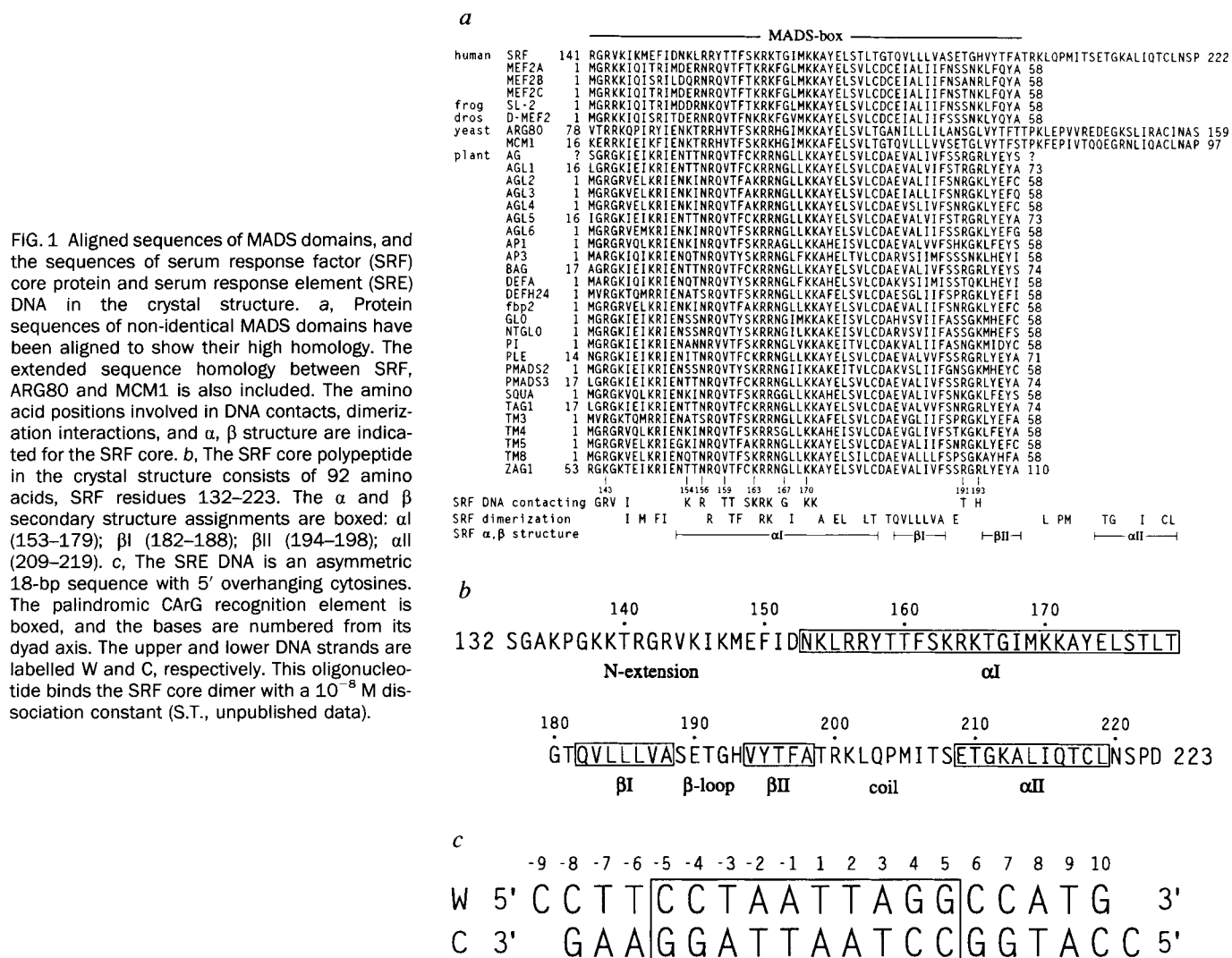
The human serum response factor is a transcription factor belonging to the MADS domain protein family with members characterized from the plant and animal kingdoms. The X-ray crystal structure of the serum response factor core in a specific-recognition DNA complex shows that the functions of DNA binding, dimerization and accessory-factor interaction are compactly integrated into a novel protein unit. The intrinsic and induced conformation of the serum response element DNA is the principal DNA feature recognized in the specific complex.

THE human serum response factor (SRF) is a ubiquitous nuclear protein that is important for cell proliferation and differentiation¹. SRF function is essential for transcriptional regulation of numerous growth-factor-inducible genes such as *c-fos* oncogene and muscle-specific actin genes (for review see ref. 2). Although maintained at a constant level for these cases³, an increase in SRF activity is required for interleukin (IL)-2 receptor α -chain expression during T-cell ontogeny⁴. A core domain of approximately 90 amino acids is sufficient for the activities of DNA binding, dimerization and interaction with accessory factors⁵. Within the SRF core is a DNA-binding region, designated the MADS box⁶ (Fig. 1a), which shows extensive sequence homology with many eukaryotic regulatory proteins. Among

these are MCM1, the regulator of cell type-specific genes in fission yeast², DSRF, a *Drosophila* trachea development factor⁷, the MEF2 family of myocyte-specific enhancer factors⁸, and the Agamous and Deficiens families of plant homeotic proteins⁶. SRF homodimer binds specifically to the serum response element (SRE), a genomic sequence first identified in the *c-fos* promoter⁹. The SRE contains a 10-bp consensus CC(AT)₆GG called the CA₆G box found upstream of all SRF-regulated genes¹⁰.

Full transcriptional activity from the *c-fos* SRE requires accessory factors that specifically bind to the SRF-SRE complex³. Some of these coactivators or ternary complex factors (TCF), Elk-1, SAP-1a, b, SAP-2, and ERP-1, have been isolated and identified as members of the Ets family of transcription factors and oncogene products^{11,12}. Others known to modify SRF DNA binding are the HTLV-1 Tax and the homeodomain Phox1 (refs

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13, 14). The transcriptional activity of the ternary complex is modulated by constitutive and growth-factor-dependent phosphorylation by means of sites on SRF and the accessory proteins^{2,15}. The target of SRF may be the basal transcription factor TFIIF, as indicated by protein-protein interaction assays¹⁶.

Here we report the first three-dimensional structure of a MADS family protein, the human SRF core in complex with a specifically recognized SRE DNA. The SRF core-SRE structure reveals that the multiple functions of DNA binding, dimerization and recruitment of collaborating factors are integrated into a single protein domain of new design. The SRF structure can explain the DNA-binding specificity not only of its own site, but also of those for the related SRF (RSRF) and yeast MCM1 proteins. The binding sites for the SRF and MCM1 accessory proteins, as well as the region of MCM1 responsible for transcription activation, can be visualized on the structure by using the results of previous mutagenesis studies.

Structure determination

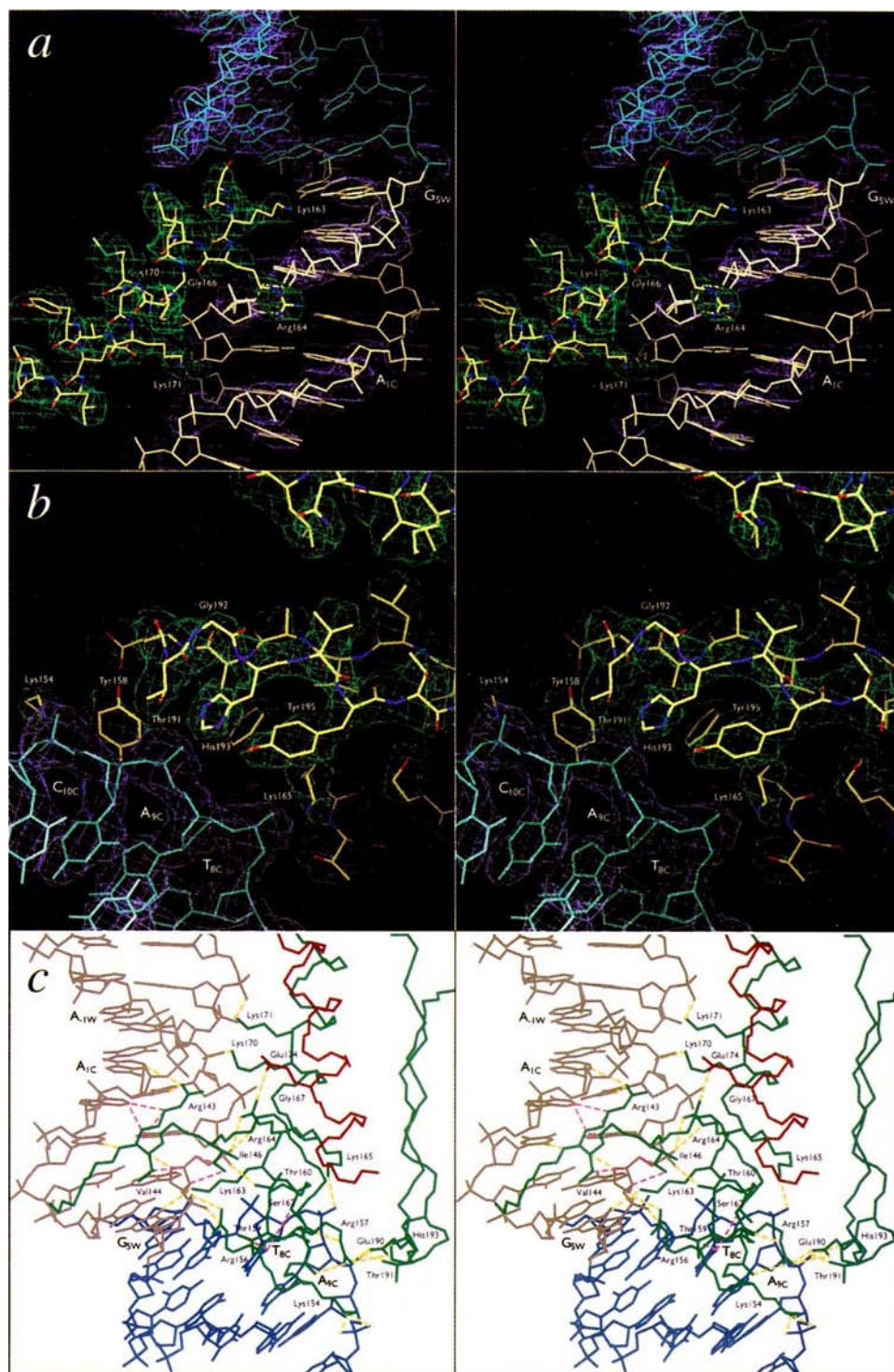
The SRF core sequence spanning amino acids 132–223 was expressed in bacteria, purified and cocrystallized with a synthetic 18-bp DNA duplex from an upstream activating sequence found in the α -cell-specific *Mfa1* gene of the yeast *Saccharomyces cerevisiae* (Fig. 1). This DNA contains a SRE consensus CC(AT)₆GG sequence that is identical to a GArG element found associated with a human α -actin gene¹⁷. An unpaired cytosine was included 5' of each terminal C·G base pair to promote

crystallization successfully through triple strand formation. The X-ray structure of the complex was solved by multiple isomorphous replacement (MIR) using phase information from one mercury and four iodine derivatives, and yielding a final *R*-factor of 22.8% to 3.2 Å (Table 1a, Fig. 2).

SRF core-DNA complex

The SRF core is a homodimeric protein composed of monomers of relative molecular mass (*M_r*) 10.4K. The molecular two-fold axis of the protein is coincident with the axis relating halves of the palindromic DNA (Fig. 3). The primary DNA-binding element is an antiparallel coiled coil of two amphipathic α -helices, one from each subunit (Figs. 2a and 4a). It is aligned approximately parallel to the minor groove in the centre of the CArG sequence, and is perpendicular to the protein dimer two-fold. The DNA molecule wraps around the coiled coil allowing the basic amino termini of the α -helices to fit into the DNA major groove in both half-sites. The chain extending from the N-termini of these helices reaches over the DNA backbone and penetrates into the minor groove. A four-stranded, antiparallel β -sheet packs against the coiled coil face opposite the DNA and is the central element of the dimerization interface. The carboxy-terminal region of each SRF monomer adopts an irregular helically coiled structure followed by a short α -helix folded over the top of the β -sheet. Overall, the SRF core domain is organized in three tightly packed structural 'layers': the coiled coil or bottom layer, the β -sheet or middle layer, and the C-terminal region or top layer. The DNA is bent around the protein and displays

FIG. 2 The final $2F_o - F_c$ electron density, and protein-DNA interactions. **a**, The interaction of one α I helix with the CArG DNA. The map is contoured at 1.0σ with densities for protein green and DNA blue. The base pairs inside the CArG box are brown, and outside blue. Gly 167 allows the close approach of the MADS-motif coiled coil to the DNA helix, and the pairs of basic side chains of Lys 163, Arg 164 and Lys 170, Lys 171 extend from the α -helix and straddle a DNA backbone. The invariance of these residues and the high conservation of the entire coiled coil for many MADS-box sequences suggests that the orientation and fit of the MADS domain on various DNA recognition sites must be nearly constant over the species and tissue types examined. **b**, The interaction of the β -hairpin loop and α I helix with the DNA backbone. Thr 191 and His 193 of the β -loop from hydrogen bonds to the DNA phosphate at position 9, and Lys 154 and Lys 165 of the α I helix interact with the phosphates at position 10 and 8, respectively. **c**, The interface between one SRF core monomer and one DNA half-site. In addition to the interactions noted in **a** and **b**, Gly 142 and Arg 143 are embedded in the CArG box (A + T)-rich minor groove. Further basic residues in the N-extension, Arg 141, Lys 145 and Lys 147, are exposed to solvent and disordered in the electron density map. Hydrogen bonds and hydrophobic contacts are yellow and pink, respectively. **d**, Schematic summary. The amino acids of the monomer which contact the longer DNA half-site are grouped by protein secondary structural element and begin with a capital letter. Solid and broken lines indicate interactions with the major and minor groove, respectively.



unusual groove widths over its entire length in comparison to canonical B-DNA.

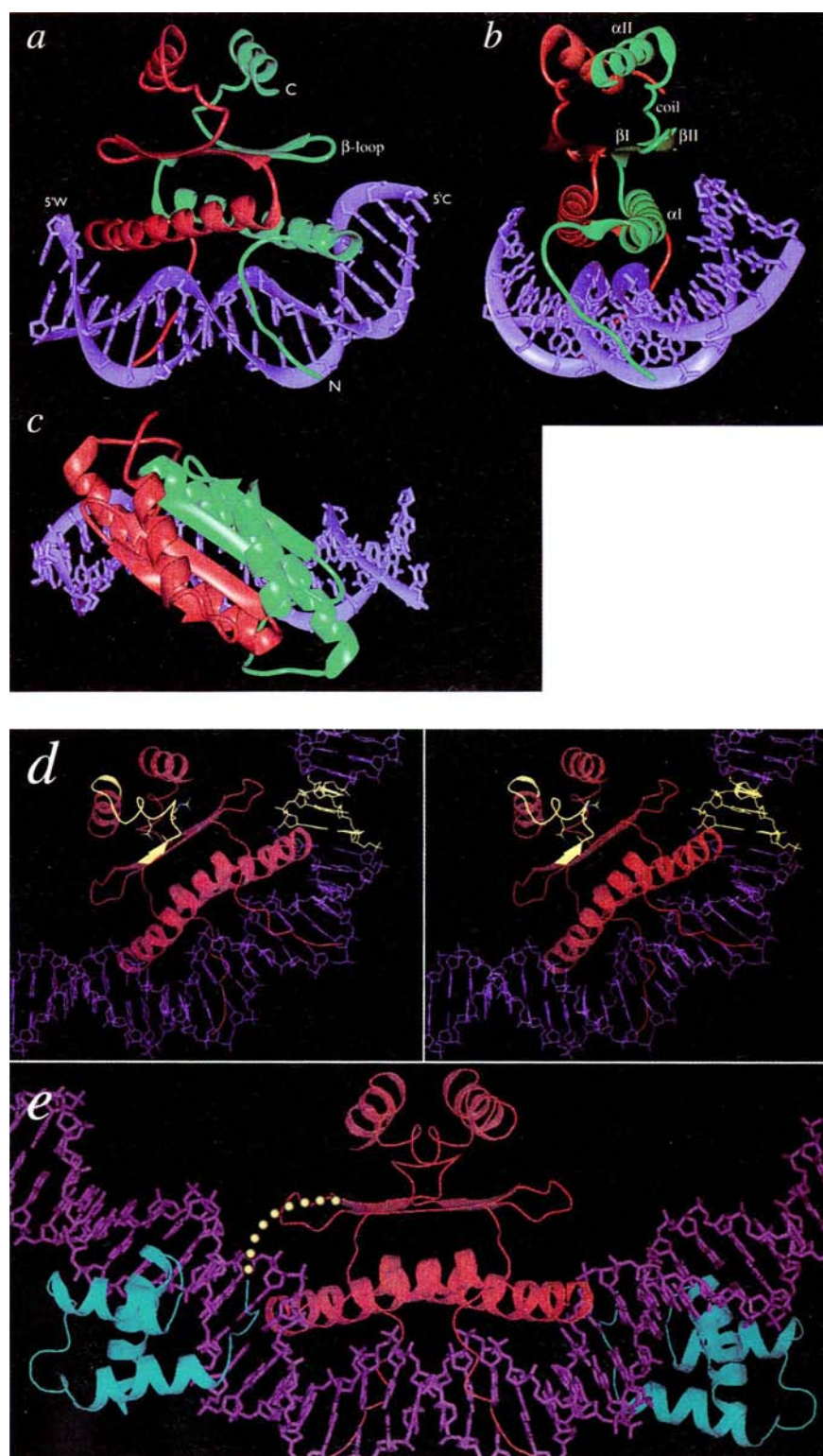
SRF core protein

The slab-like SRF core monomer is oriented in the dimer with a flat hydrophobic face parallel to the dimer two-fold axis such that identical secondary structural elements are matched between monomers (Fig. 3). The smallest SRF peptide reported that is capable of *in vitro* dimerization encompasses residues 168–222 (ref. 1), but the crystal structure shows dimerization contacts from Ile 146 to Leu 219 (Fig. 1a).

The predominant element of the DNA-binding motif is a left-handed, antiparallel coiled coil formed from the C-terminal 5.5 of 7.5 turns of an amphipathic α -helix (α I, residues 153–179;

Fig. 4a). The α I helix has a kink of 18° at Ser 162, a 16° crossing angle with its mate in the coiled coil, and is divided into two functionally distinct parts: the N-terminal third that interacts with the DNA major groove, and the C-terminal two-thirds that lies along the DNA backbone. A tight turn centred on Gly 180 links the α I helix to a highly hydrophobic β -hairpin (β I, residues 182–188; β II, residues 194–198) that runs approximately parallel to the helix axis and leaves only the first α I turn exposed. In the dimer, the two β -strands are joined in a four-stranded, antiparallel β -sheet that forms the middle layer of the structure. The inner β I strand, which contains the six consecutive hydrophobic residues VLLLVA, forms the centre of the hydrophobic core. Of the 24 residues with β conformation, 20 are completely hydrophobic. Both the α I helix and the β I strand of the MADS domain

FIG. 3 The SRF core–DNA complex and accessory factor binding. *a*, View perpendicular to the molecular two-fold and overall DNA helix axes. The two SRF core monomer α traces are distinguished in red and green; DNA is blue. The longer DNA half-site is associated mainly with the green monomer. *b*, View revealing the slab-like structure of the monomers. The difference in accessible surface area between the two isolate monomers and the dimer is $3,819 \text{ \AA}^2$ (monomers, $7,012 \text{ \AA}^2$ and $6,733 \text{ \AA}^2$; dimer, $9,926 \text{ \AA}^2$). The two monomers have highly similar conformations: the α atoms superimpose with $0.39 \text{ \AA}$ r.m.s. deviation for residues 140–219. *c*, View down the molecular two-fold axis. *d*, The SRF and SRE binding sites for the Elk-1 TCF protein. The putative regions (yellow) on the SRF core dimer (red), including side chains for Ala 198, Arg 200 and Gln 203 (ref. 5), and DNA (purple) implicated in SRF/TCF interaction are shown. In the *c-fos* SRE, the middle of the TCF binding site lies 10 bp from the CARG-box centre¹⁵ and, because Elk-1 binds to the DNA major groove⁴⁸, lies on the side opposite to SRF. The TCF linker polypeptide that connects the Ets DNA-binding domain with the SRF interaction domain can apparently span between sites because of its putative flexibility owing to a high content of glycine, proline and serine over a length of approximately 50 residues¹². *e*, Simulated complex of MAT α 2 monomeric homeodomain and MCM1 core. The DNA sites in the binary complexes of the MAT α 2 homeodomain⁴⁹ (blue) and the SRF core (red) X-ray structures were superimposed to show the relationship of the two proteins in a hypothetical ternary complex. In this simulation, no conflicts of atomic position occur between the proteins although their DNA sites overlap. A proposed initial path (yellow dots) for the putative MCM1 binding region of intact MAT α 2, a flexible region of 30 amino acids just before the C-terminal homeodomain⁴¹, would take it along the MADS-domain β II strand.



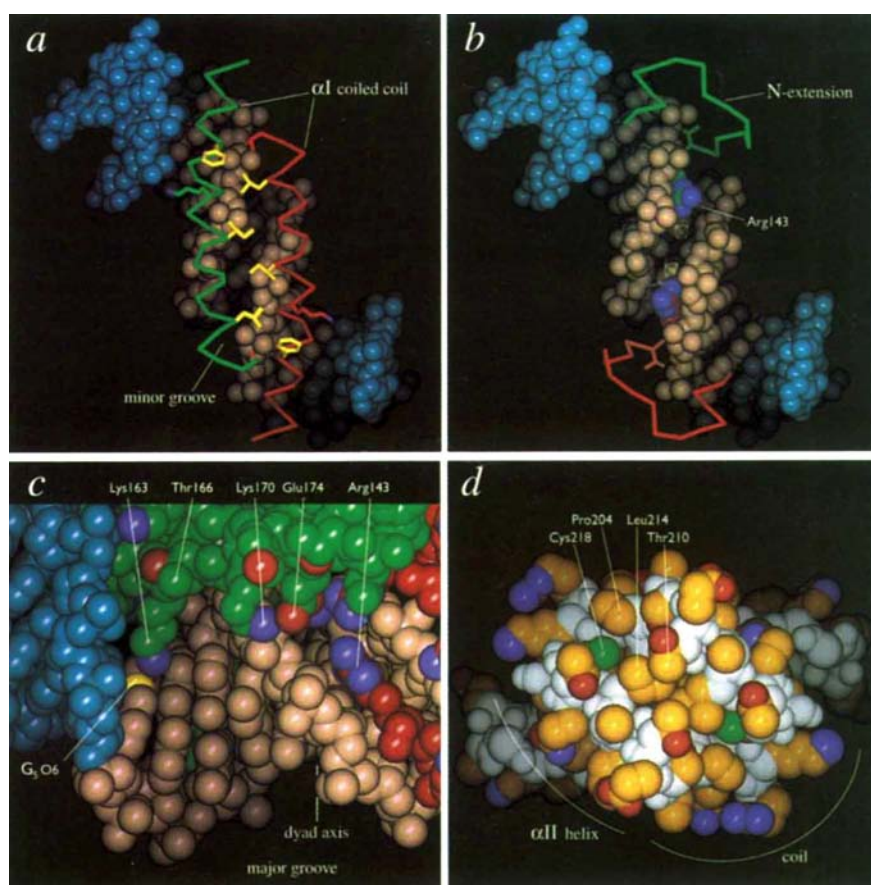
the major groove opposite has broadened to $19.5 \text{ \AA}$. As this DNA sequence may be relevant to the structure of free, bent DNA, we note that, in addition to positive base pair roll in steps adjacent to the (A+T)-rich central sequence, the TAATTA sequence itself displays a small average negative roll of -2.5° . The correlation of narrow minor groove with high propeller twist in runs of A·T base pairs observed for free DNA structures also occurs in the SRF core complex with values ranging from -7° to -17° (ref. 19). The large helical twist angles of ApA and TpA steps or overwinding in the centre of the CARG box are

partly compensated for by the ApT step and the outer underwound base steps in this highly distorted B-DNA.

SRF core–DNA interactions

In the crystal, the two-fold axis of the SRF core superimposes with the dyad of the SRE CARG box and, as a consequence of the choice of DNA, the two dimer subunits contact half-sites of different length. However, the protein–DNA interactions within the CARG box are common to both monomers except where noted (Fig. 2*d*). Each α I helix has two pairs of successive, highly

FIG. 4 Structural features of the SRF core-DNA complex. The DNA is shown in space-filling representation with CArG box brown. *a*, The SRF DNA-binding motif is an antiparallel coiled coil of two α -helices. The hydrophobic side chains (yellow) of these two α helices (red and green) have heptad repeat positions similar to the leucine sites of a leucine zipper. Compared to the parallel coiled coil in the GCN4-bZIP⁵⁰, the leucine positions (d-site) contain Phe 161, Ile 168 and Leu 175, and the valine positions (a-site) contain Lys 165, Ala 172 and Thr 179. *b*, The N-extension links Arg 143 to the N-terminal turn of the α helix. The Arg 143 guanidinium group visible in the narrow minor groove and the Val 144 side chain anchoring the protein to a deoxyribose group are shown in each half-site. A probable water molecule, represented by a peak of electron density 5σ above background in a $F_o - F_c$ map (yellow), could link Lys 171 to Arg 143. *c*, The major groove of the CArG consensus sequence is accessible. The interaction of Lys 163 with O6 of base G₅ is the only hydrogen bond made to the DNA in the major groove per half-site. In contrast, the minor groove of the CArG box is filled with Arg 143. Phox1 homeodomain protein may form a ternary complex with SRF by binding to the open major and adjacent minor grooves¹⁴. Thr 166 is not conserved in SRF homologues RSRF and MCM1, and a larger side chain at this position would enter the major groove and perhaps help the specification of the base recognized at DNA position 3. *d*, The solvent-exposed surface of the coil and all structural layer is predominately hydrophobic. Main-chain atoms (white), side-chain oxygen and nitrogen atoms (red and blue), cysteine sulphur (green) and side-chain hydrophobic groups (yellow) are distinguished. Deletion of the last turn of the α helix is sufficient for loss of the *in vivo* activation function of the highly homologous MCM1 protein. For both copies of α II, the last residue in



helical conformation is Leu 219, and the extended NSPD C-terminal sequence seen for one monomer is highly disordered in the other.

conserved basic side chains that straddle a phosphodiester chain. The pair nearest the molecular dyad axis, Lys 170 and Lys 171, interact with the DNA backbone. The Lys 171 side chain projects into the minor groove and is surrounded by the phosphates of bases T₂ and A₋₃ and the carboxylate of Glu 174 in the same monomer. This negatively charged pocket determines the position of Lys 171 for which no hydrogen bonds are apparent. The Lys 170 side chain forms hydrogen bonds to the phosphate of base T₂ on the major groove side and to the Glu 174 carboxylate of the adjacent monomer. The second pair of basic residues, Lys 163 and Arg 164, two α -helix turns away from Lys 170 and Lys 171 and near the helix centre, have been shown by mutagenesis to be essential for SRF binding to the *c-fos* SRE²⁰. Lys 163 points straight into the major groove and forms a hydrogen bond to O6 of G₅ at the extremity of the CArG box, explaining the protection of this base from dimethyl sulphate modification²¹. The N7 of G₄ was also found inaccessible, and in the half-site with greatest DNA bending, it also has a hydrogen bond to Lys 163. In both half-sites, this position is excluded from solvent by the methylene groups of Lys 163 and by base A₃ or A₋₃ rolled into the adjacent guanine. The Arg 164 side chain forms hydrogen bonds with N₁ and N₈ atoms to the phosphate of G₄ on the minor groove side of the DNA backbone. The side-chain orientation is stabilized by a hydrogen bond to the side chain of Glu 174 from the other monomer. Thr 160 on the preceding α -helix turn has a hydrogen bond to the phosphate of G₄ on the major groove side and, one turn further, the guanidinium group of Arg 156 stretches across the DNA chain and forms hydrogen bonds to the G₅ phosphate. The close approach of the α I helix to the DNA relies on invariant Gly 167, which lies between the Lys 170, Lys 171 and Lys 163,

Arg 164 pairs on the intervening turn of α -helix, to be in van der Waals contact with the A₃ phosphate.

A third basic residue, Arg 157, shown to be essential for DNA binding²⁰, unexpectedly points away from the DNA and bridges from the side chain of Glu 190 in the β -loop to the main chain of Asp 152 and Ile 151 within the same monomer (Fig. 2c, d). All three guanidinium nitrogens are essential for these interactions as the conservative substitution to lysine abolishes DNA-binding activity completely²⁰. Arg 157 guides the N-extension emanating from the α I helix in a U-turn back towards the minor groove so that it enters the DNA helix at base pair G · C₄, allowing the main-chain amide of Arg 143 to form a hydrogen bond to the T₃ O2 atom. The Arg 143 side chain lies in an extended conformation buried in the narrow minor groove packed between deoxyribose rings and against the C2 atoms of two adenine bases. One guanidinium N₁ is simultaneously hydrogen bonded to N3 of A₁ and O2 of T₂. Replacement of the A · T base pairs with G · C would not only widen the minor groove, but the guanine N2 atoms would block the observed arginine orientation. Gly 142 allows the Arg 143 C α atom to be embedded in the groove. Extensive hydrophobic interaction immediately adjacent to Arg 143 occurs between Val 144 and Ile 146 and the G₄ deoxyribose.

The SRF core subunit bound to the longer DNA half-site contacts DNA outside the CArG box. These interactions with the C-strand phosphates of bases C₁₀A₉T₈ are across the major groove from those described for the W-strand (Fig. 2c, d). Lys 165 in the middle of the α I helix extends to the phosphate group of T₈ in the opposite direction to the side chain of Lys 163. A similar pairing occurs for Lys 154 and Arg 156, where Lys 154 spans across the major groove to the phosphate of C₁₀. The

second layer of the structure has only two direct contacts with DNA: Thr 191 and His 193 in the β -loop contact the phosphate group of A₉. The other half-site has none of these interactions because the DNA fragment stops at base pair C · G₈. The importance of the C₁₀ phosphate is seen in SRF core gel binding assays in which symmetric oligonucleotides of 10, 16 and 20 bp with a central CArG box reveal that, in conditions where the 20-bp fragment yields a 10⁻⁹ M dissociation constant, the shorter fragments are unbound (S.T., unpublished data).

A convincing sequence preference outside the CArG box for T₈ has been detected by selective binding of DNA oligonucleotides from random sequence pools²² and examination of natural SRE sequences⁹. The crystal structure shows that the 5-methyl group of T₈ makes hydrophobic contacts with the Thr 159 C_γ and Ser 162 C_β atoms (Fig. 2c, d). No base except G₅ makes such an obvious major groove-specific contact.

Implications for protein–DNA interactions

Runs of six A · T base pairs, as at the centre of the CArG sequence, produce a narrow minor groove, and can be specifically recognized by DNA-binding drugs and protein¹⁹. As noted previously, p53, 434 and 434 *cro* repressor, HNF-3 and MATα2 have arginine side chains that penetrate the DNA minor groove, forming hydrogen bonds to phosphates or to thymidine O2 and adenine N3 atoms²³. For SRF core, Arg 143 is entirely buried in the minor groove, making direct contacts with all three bases in the TTA half-site sequence (Figs. 2c, d and 4b). Thus the N-extension provides a recognition element for the (A + T)-rich, narrow minor groove. The interactions of Gly 142, Arg 143 and Val 144 of SRF are almost identical to those of the GRP sequence seen in the Hin recombinase–DNA complex²⁴ and of HMG-I/Y derived peptides²⁵. In SRF core, Lys 171 of the complementary coiled coil α -helix is additionally positioned over the arginine, and interacts electrostatically with both phosphodiester chains across the narrow groove. With regard to DNA bending, the helix axis of the AT region is not absolutely straight as might be expected from B-DNA structures, but bends overall by 13° toward the minor groove and protein. The negative roll of the AT-tract base pairs accounts for approximately one-sixth of the overall bend angle, with the positive roll of the flanking sequences accounting for the remaining 59°.

The X-ray structure of the CArG boundary sequence for one half-site flanked by A · T base pairs is known from the decamer CATGGCCATG²⁶. The GGCC sequence in the free decamer is smoothly bent 23° through base pair roll and major groove compression, as against 32° for the analogous bend in the SRF core complex. The 'pull' of the protein on the DNA by means of contacts with the phosphates of bases C₁₀A₉T₈ may account for at least part of the additional curvature seen in the protein–DNA complex, as these interactions contribute to the binding energy. The site permutation gel assay for DNA bending indicates that the target site DNA is more highly bent with SRF bound than it is without, although this might be expected because SRF stabilizes the DNA in the bent conformation, and the CC and GG sequences are not spaced by one-half of the helix repeat²⁷. The similarity of the CArG box to sequences known to be bent²⁸ suggests that the SRF protein recognizes an intrinsic bend in the DNA which has propensity to bend further in the protein–DNA complex.

Related SRF proteins

Related SRF proteins (RSRF; for example, MEF2C, Fig. 1a) share high homology with SRF over the N-terminal half of the MADS domain, and recognize the sequence CTA(AT)₄TAG as compared to the SRE CC(AT)₆GG, as determined by *in vitro* site selection²⁹. As all amino acids except one contained in the N-extension and α I helix responsible for DNA interaction are conserved, it is likely that the orientation of the protein on the DNA and the details of interaction are essentially identical. The SRF Thr 166 to RSRF Phe 166 sequence difference appears to

contribute to the selection of base pair A · T₃. In SRF core, Thr 166 is located over the major groove but makes no contact with the DNA, and the side chain of phenylalanine at this location can be orientated to make hydrophobic contact with the 5-methyl group of a thymidine at W-strand position 3 (Fig. 4c). Improved discrimination between the RSRF and SRF DNA target sites is seen for a truncated SRF molecule (METcore: SRF sequence with initiator methionine at position 141) incorporating Phe 166 (ref. 30).

The selection of base pair T · A₄ by RSRF cannot depend on major groove contacts as the only residues nearby are Lys 163 and Phe 166, which are too far away to contact the T₄ 5-methyl group. However, in the minor groove, A₄ can be distinguished from the other bases by the lack of a heterocyclic atom at C2. Based on the SRF structure, an RSRF N-terminal methionine would be located over A₄ and could possibly specify this adenine by means of hydrophobic interaction with its C2 atom. In accord with this proposal, an SRF core having an N-terminus chosen to match that of RSRF is reduced in affinity for the SRF consensus site but increased for the RSRF-selected sequence³⁰. This interpretation and the RSRF-selected sequence rely on the continued presence of the N-terminal methionine after *in vitro* expression. The amino acids N-terminal to Gly 142 in SRF are unlikely to contribute to DNA-binding specificity because Thr 140 and Arg 141 do not make sequence-specific contacts, and the remaining eight residues are disordered.

Lys 154 is unlikely to be a major determinant for the selection between RSRF and SRF sites by means of direct base contact as suggested previously³⁰ because it is hydrogen bonded to the C₁₀ phosphate five base pairs from the end of the CArG box. The METcore mutants containing Glu 154 show most significantly a loss of affinity for CC(AT)₆GG, which would be expected from placing a negatively charged side chain close to the DNA phosphate backbone. Lys 154 may play a role based on differences in DNA conformation. The CTA(AT)₄TAG site may have a different backbone trajectory (for example, it may be less bent) in the site extremities which avoids glutamate–phosphate proximity or alternatively links these groups by means of a divalent cation.

DNA sequence specificity of MCM1

The yeast MCM1 protein has 72% sequence identity to SRF over the MADS box³¹, yet binds the variant consensus sequence, CC(C/T)AA(A/T)NNGG, as derived by *in vitro* site selection³². The requirement for A or T at position 3 has changed to C or T, and, as for RSRF, seems to depend on the residue at SRF position 166. For MCM1, the histidine side chain could bridge by means of a water molecule to a C-strand purine N7 atom, and thereby account for the W-strand preference for pyrimidine. A second important difference between the consensus sequences is the lower requirement for A or T in the centre of the MCM1 binding site. MCM1 must then recognize a wider minor groove than SRF with extracyclic N2 groups protruding from some bases, which it does with the sequence ERR instead of the GRV of SRF. The glutamic acid almost certainly eliminates the arginine conformation observed in SRF. One or both of the MCM1 arginine side chains may interact in the groove using only their guanidinium moiety, perhaps in the p53-like conformation noted above.

The DNA sequence requirements for MCM1 are more complicated than for SRF. At least two types of sites must be recognized, distinguishing α and α -cell type specific genes, and yielding functionally distinct complexes. Based on consensus upstream activating sequences (UAS) from α - and α -specific genes, representative sites for each with minimal differences were derived and shown to function *in vivo* as expected³³. From the α -specific P-element TTTCCTAATTAGGAAA and α -specific P'-element TTTCCTAATTAGTGTC (P element invaded by the Q box on the right) sequences chosen, it is clear that G · C base pairs in the central six positions cannot be the crucial difference, nor can

the exchange of the GG sequence to GT because the central 10 bp of the α -specific *MATa1A* sequence conforms perfectly with this P element through the GG sequence. We have observed by primer extension assay that, for the highest affinity binding, the P' sequence must extend a minimum of 10 bp from the pseudo-palindrome centre on the Q-box side but the other side of P' and the P sequence must cover only 8 bp (S.T. and T.J.R., unpublished data). Based on the SRF core structure, it is likely that α -specific sites do not interact with the β -loop or Lys 154, but α -specific elements do make these interactions on the Q-box side. The preferred P-element sequence of AAA versus the Q-box sequence at positions 6–8 appears to be the significant determinant.

Activation by MCM1/SRF core

The sequence homology between SRF and MCM1 continues for 24 amino acids beyond the C-terminal boundary of the MADS box (Fig. 1a), and corresponds to the third structural layer of the SRF core. The MCM1 core is sufficient for DNA binding and transcriptional activation when bound to α -specific UAS elements in α -cells³⁴. Chimaeric molecules constructed from MCM1 N-terminal and SRF C-terminal halves can substitute for MCM1 core and maintain the essential and intrinsic activation function *in vivo*^{5,35}. The solvent-exposed surfaces of the third-layer coils and α II helices are predominantly hydrophobic as a consequence of outward-facing alanine and threonine side chains and the presence of two pairs of interacting side chains linking monomers: Thr 210-Leu 214 and Pro 204-Cys 218 (Fig. 4d). Deletion of only the C-terminal turn of the α II helix is sufficient to eliminate the activation activity³⁶.

The additional protease sensitivity of MCM1 when bound to an α -specific as compared to an α -specific UAS suggests that MCM1 undergoes a DNA-induced conformational change which enables transcription activation from α -specific sequences³⁷. However, the additional protease cleavage site is C-terminal to the core, so its structure is unknown. The conformational difference may be linked to a structural alteration of the β -loop dependent on DNA binding, or simply to a difference in the proximity of the DNA to C-terminal sequences that affects their arrangement.

Interaction with accessory factors

Ternary complex factors (TCF), Elk-1 and SAP-1, depend on SRF association to bind SRE¹⁵. These Ets-family oncoproteins have three regions of homology corresponding to DNA binding, SRF binding and transcription activation. Mutagenesis of SRF has indicated that Ala 198, Arg 200 and Gln 203 are directly in the protein-protein interaction interface as they are sufficient to endow the ARG80 homologue of SRF with the ability to bind Elk-1 (ref. 5). The SRF structure reveals that Arg 200 and Gln 203 point into solution from the helical coil connecting the β II strand and the α II helix (Fig. 3d). Further mutagenesis implicates a larger region including the entire β II strand and part of the α II helix³⁸.

Yeast MCM1 also recruits accessory factors similar to the TCF proteins, and chimaeric constructs demonstrate that residues corresponding to SRF 198, 200 and 203 in β II are also important for MCM1-STE12 recognition⁵. Point mutations of MCM1 indicate that the entire β II strand and coil preceding the α II helix are involved in STE12 and MATa1 binding, and that STE12 interaction also involves a valine at position 177 in the C terminus of the α I helix adjacent to the coil region³⁹. The MCM1 histidine residues at positions 158 and 166, which are both likely to contact DNA in the binary complex, yield mutants with decreased ability to activate transcription from α -specific UAS elements. However, these variants do not always have a lower capacity to form MATa1 complexes, which implies that MCM1/DNA conformation in the binary and ternary complexes may be somewhat different.

MATa2, a dimeric homeodomain protein, forms a complex with MCM1 in yeast α -cells that represses α -cell specific genes. *In vitro*, MCM1/MATa2 bind α -specific UAS elements with 500-fold cooperativity⁴⁰. As with TCF/SRF, the coil segment following the MADS-box β II strand of MCM1 has been shown by mutagenesis to affect MATa2 binding³⁹. Our model building suggests that the region of MATa2 responsible for association with MCM1 (ref. 41) would be suitably located to contribute an additional antiparallel strand to the MADS-domain β -sheet of MCM1 (Fig. 3e).

Conclusion

Site-specific DNA recognition by SRF depends almost exclusively on intrinsic and inducible conformational properties of DNA: selection of a narrow minor groove characteristic of (A+T)-rich sequences, and detection of shape based on GG flanking sequences that favour bending into the major groove. A new structural motif is used for DNA interaction, namely an antiparallel coiled coil of α -helices that lies flat on the DNA minor groove. Recognition of the consensus binding site by interaction with a pattern of hydrogen-bond donor and acceptors and/or hydrophobic groups in the major groove is limited to a single specific interaction per half-site. Other sequences of MADS-box proteins and their DNA-binding sites indicate that additional base interactions may compensate for DNA with a different propensity for bending. For MCM1, increased interaction with the bases may allow sequence-dependent, DNA-bending preferences to produce functionally distinct complexes. The ability of SRF to recognize DNA bend sites apparently allows it to act as an adapter for accessory proteins which cannot alone adequately recognize their DNA target sites. The exposed β -strand in the MADS motif may be an important element recognized by the flexible combining regions of accessory factors. Further protein-protein interaction sites may extend to the structural elements above and below the β -sheet. It will be of interest to discover how the overall architecture of these complexes, including the effect of DNA bending and multiple protein factors, contributes to transcriptional regulation.

Note added in proof: As proposed in this work, analysis of mutants of the SRF, MEF2, and MCM1 DNA-binding domains presented by Nurrish and Treisman⁵¹ demonstrates the importance of SRF positions 140 and 141 as determinants of DNA-binding specificity. □

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LETTERS TO NATURE

Efficient photodiodes from interpenetrating polymer networks

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THE photovoltaic effect involves the production of electrons and holes in a semiconductor device under illumination, and their subsequent collection at opposite electrodes. In many inorganic semiconductors, photon absorption produces free electrons and holes directly¹. But in molecular semiconductors, absorption creates electron-hole pairs (excitons) which are bound at room temperature², so that charge collection requires their dissociation. Exciton dissociation is known to be efficient at interfaces between materials with different electron affinities and ionization potentials, where the electron is accepted by the material with larger electron affinity and the hole by the material with lower ionization potential³. A two-layer diode structure can thus be used, in which excitons generated in either layer diffuse towards the interface between the layers. However, the exciton diffusion range is typically at least a factor of 10 smaller than the optical absorption depth, thus limiting the efficiency of charge collection³. Here we show that the interpenetrating network formed from a phase-segregated mixture of two semiconducting polymers provides both the spatially distributed interfaces necessary for efficient charge photogeneration, and the means for separately collecting the electrons and holes. Devices using thin films of these polymer mixtures show promise for large-area photodetectors.

Conjugated polymers have been used for thin-film semiconductor devices, including transistors⁴ and light-emitting diodes (LEDs)⁵, and chemical modification of the polymer structure has allowed control of energy gap, electron affinity and ionization potential. The poly(*p*-phenylenevinylene)s, PPVs, are good hole-transporting materials and are widely used. The addition of cyano groups to a dialkoxy derivative of PPV forms the CN-PPV shown in Fig. 1, and this increases the ionization potential and electron affinity by ~0.5 eV, giving better electron injection and transport properties⁶. The availability of these electron- and hole-transporting polymers has allowed the fabrication of two-layer LEDs, where the electron and hole currents are controlled by the interface formed between PPV and CN-PPV⁷.

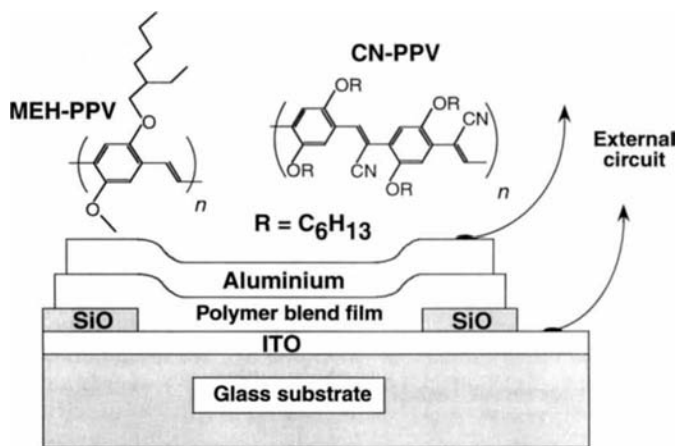
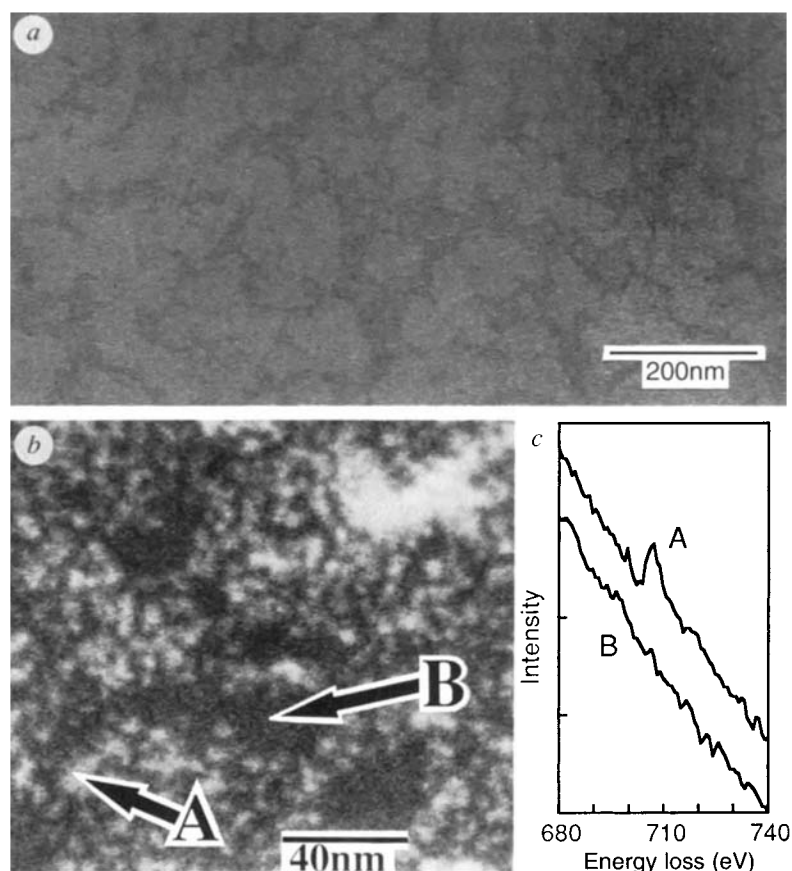


FIG. 1 Schematic diagram of the polymer photodiode, showing the two polymers used to form the interpenetrating network (ITO, indium tin oxide). The devices were fabricated by spin-coating various ratios of the polymer mixture from solution onto ITO-coated glass substrates, on which SiO insulating strips had been previously evaporated. The strips define the active area of the device and prevent short-circuits when contact is made to the electrodes. The films were annealed *in vacuo* at 100 °C for several hours to remove any residual solvent or surface contamination. Metal contacts were subsequently evaporated onto the film without breaking the vacuum.

We report here work on mixtures of a soluble PPV derivative, poly(2-methoxy-5-(2-ethyl)hexyloxy-*p*-phenylenevinylene), MEH-PPV⁸, and CN-PPV. It is well known that mixtures of polymers tend to phase segregate on account of the low entropy of mixing⁹. To investigate the phase segregation of our polymer mixtures we have used a combination of transmission electron microscopy (TEM), scanning transmission electron microscopy (STEM) and parallel electron-energy-loss spectroscopy (PEELS). Thin films (~100 nm) of the mixtures were produced by spin-coating from a solution in chloroform onto a glass slide. We have used measurements of optical absorption to show that FeCl₃ does not dope CN-PPV but can oxidatively dope MEH-PPV throughout the thickness of these films, forming sub-gap absorption bands at 0.6 and 1.6 eV, characteristic of doped polymers of this type¹⁰. This makes it possible to stain the MEH-PPV selectively. We see clear evidence for phase separation for the whole range of compositions studied; we show here micrographs for compositions of MEH-PPV:CN-PPV of 7:1 and 1:10 (by weight) as these produce particularly clear images. The TEM bright-field image, Fig. 2a, and a STEM annular dark-field image of a mixture, Fig. 2b, clearly demonstrate that there is phase separation on the scale of 10–100 nm, as expected for films of this thickness. Performing PEELS with STEM allows

FIG. 2 *a*, Bright-field transmission electron micrograph demonstrating the presence of a phase-segregated, interpenetrating network of stained MEH-PPV (dark regions) and CN-PPV (light regions). In the annular dark-field STEM image (*b*) we associate the light regions with MEH-PPV, which is verified by the characteristic iron L-edge peak in the energy-loss spectrum (*c*) measured at position A. The dark regions do not contain iron, as shown by the spectrum from position B. Micrograph *a* was obtained from a 1:10 mixture (by weight) of MEH-PPV and CN-PPV, and micrograph *b* from a 7:1 mixture. Films were spin-coated onto glass substrates, and floated off in water onto 3-mm-diameter gold electron-microscope grids. The samples were then stained using 0.5 wt% FeCl_3 solution in methanol or methanol/chloroform.



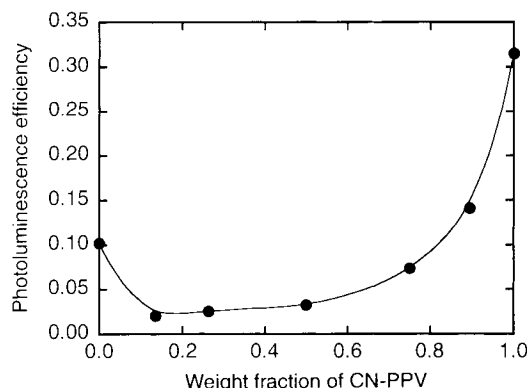
the acquisition of energy-loss spectra from areas ~ 1 nm in size. From the spectra in Fig. 2c we see that iron is present only in the lighter regions of the dark-field image, showing the presence of MEH-PPV in these regions and its absence in the dark regions. The details of the microstructure are best seen in the TEM image, Fig. 2a, which shows evidence for formation of interpenetrating networks within the plane of the film.

Evidence for photoinduced charge transfer is provided by quenching of photoluminescence. Blends of MEH-PPV with the electron-acceptor fullerene, C_{60} , have been extensively investigated^{11,12}, and show very efficient quenching (a factor of 10^4 for a blend of composition 1:1 by weight¹²), as expected for intimate mixing of donor and acceptor species. Figure 3 shows the absolute photoluminescence quantum efficiencies of a range of MEH-PPV/CN-PPV mixtures, measured using an integrating sphere¹³. We measured values for MEH-PPV and CN-PPV of

10% and 32% respectively, similar to values reported previously¹³, and found quenching to a level of around 2% to 5% for the mixtures in the composition range 1:4 to 4:1 by weight. This substantial but incomplete quenching is as expected¹⁴ for an exciton diffusion range of 10 nm, given the scale of phase segregation observed in the electron micrographs shown in Fig. 2. We propose that excitons are dissociated at the dispersed interfaces by transfer of electrons to the CN-PPV and of holes to the MEH-PPV.

Photovoltaic cells were prepared with a polymer layer sandwiched between electrodes with different work functions, as used in polymer-based LEDs (Fig. 1). Figure 4a shows the current-voltage characteristics of a polymer-mixture device, both under illumination of 0.15 mW cm^{-2} at a wavelength of 550 nm, and in the dark. In the dark, the device exhibits a rectification ratio of 10^3 at ± 3.5 V. Under illumination, devices of this type show

FIG. 3 The absolute photoluminescence efficiency of the polymer mixture (prepared as thin (100 nm) films on quartz substrates) plotted as a function of composition. Excitation was at a wavelength of 457.9 nm. The line shown is a guide to the eye. The photoluminescence spectra of the mixtures are a combination of features due to pure MEH-PPV⁸ and pure CN-PPV emission⁷.



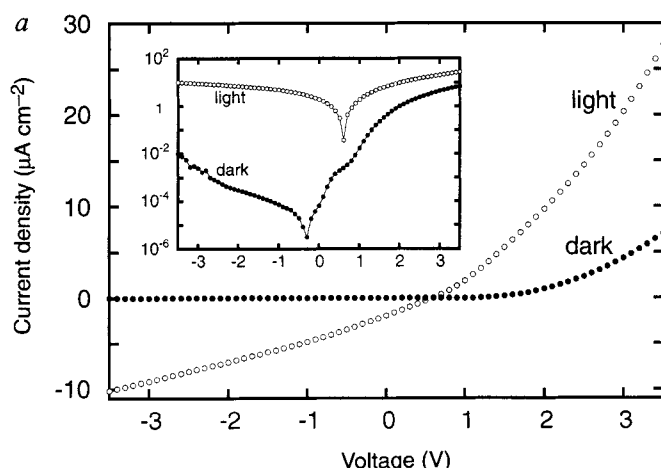
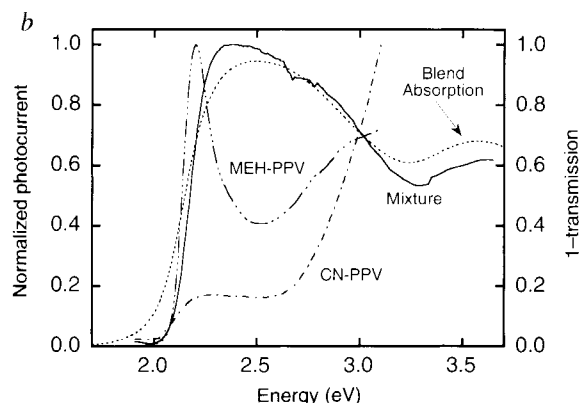


FIG. 4 a, The current-voltage characteristics of an ITO/(MEH-PPV/CN-PPV mixture)/Al device in the dark, and under illumination at a wavelength of 550 nm and intensity of 0.15 mW cm^{-2} . (Inset, the same data, with a logarithmic current axis). b, The spectral responses of the short-circuit photocurrent through an ITO/polymer/Al device. Currents have been corrected for the lamp-monochromator system response and scaled to give a peak current of unity for all devices. Intensities of



incident light were of the order of 0.1 mW cm^{-2} . The absorption spectrum of the MEH-PPV/CN-PPV film, presented as $(1 - \text{transmission})$ for a film of the same thickness, is also shown. The polymer mixture here consisted of equal masses of the two components, but very similar results were found for mixtures of different ratios, including 7:1 and 1:10 (by weight).

a strong photoresponse, with open-circuit voltages of 0.6 V, and short-circuit currents which correspond to quantum efficiencies of up to 6%. Under forward or reverse bias, the quantum efficiencies rise rapidly, reaching 15% at a reverse bias of 3.5 V, 40% at 10 V, and considerably higher values under forward bias. These performance figures are very much better than those for similar devices made with aluminium electrodes and either MEH-PPV or CN-PPV alone. We found quantum yields of 0.04% for the short-circuit photocurrent at the peak response energy (2.2 eV) in the devices made with MEH-PPV, and of the order of 10^{-30} for those made with CN-PPV at 2.8 eV. (Note that better efficiencies are seen for single-polymer devices if cathodes with lower work functions are employed, such as Ca and Mg^{15,16}.)

The spectral response of photodiodes of this type provides detailed information about the device operation. Figure 4b shows the spectral dependence of the short-circuit current for devices made with the polymer mixture and with the individual polymers. We find that the spectral response of the mixture device matches the absorption (also shown in the figure) very closely, and we consider that this is excellent evidence for efficient charge generation and transport to the device electrodes. In contrast, the devices made with the individual polymers show more complicated spectral responses^{15,17,18}. For example, the peak in response for PPV at the absorption edge is attributed to electron trapping in the photogeneration region¹⁵.

The properties of these phase-separated polymer-mixture devices are essentially different to those of devices made with polymers blended with small molecules, as exemplified by the conjugated polymer/ C_{60} blends^{11,12}. The latter show very efficient photoinduced charge transfer, but only one of the photo-generated charges is mobile. Such blends can be used to provide unipolar transport, as exploited in some of the organic photoconductors used in xerography¹⁹. In contrast, the morphology of the phase-separated materials which we report here allows both photogenerated electrons and holes to be transported to the electrodes before recombination occurs. Collection of both carrier types is necessary for operation in a photovoltaic mode.

Polymer morphology has been used to control electronic properties in other ways. For example, Berggren *et al.*²⁰ showed that the self-organizing property of polymer mixtures can be used in polymer LEDs to obtain voltage-controlled colour sources. Also, Yang and Heeger²¹ have used a conducting polyaniline network as a 'grid' in a polymer-based solid-state triode. An alternative approach to producing photodiodes was adopted by O'Regan *et al.*²² who used sintered rutile coated with a ruthenium dye to achieve a large surface area for efficient charge dissociation. The phase-separated polymer photodiodes that we have presented here have not yet been optimized for use for solar energy conversion. However, we foresee particular interest in their use for large-area, flexible photodetectors, as required for example in medical imaging²³.

Note added in proof: Similar measurements of photoelectric properties have recently been performed by Yu and Heeger²⁴. □

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Climate response to increasing levels of greenhouse gases and sulphate aerosols

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CLIMATE models suggest that increases in greenhouse-gas concentrations in the atmosphere should have produced a larger global mean warming than has been observed in recent decades, unless the climate is less sensitive than is predicted by the present generation of coupled general circulation models^{1,2}. After greenhouse gases, sulphate aerosols probably exert the next largest anthropogenic radiative forcing of the atmosphere³, but their influence on global mean warming has not been assessed using such models. Here we use a coupled ocean-atmosphere general circulation model to simulate past and future climate since the beginning of the near-global instrumental surface-temperature record⁴, and include the effects of the scattering of radiation by sulphate aerosols. The inclusion of sulphate aerosols significantly improves the agreement with observed global mean and large-scale patterns of temperature in recent decades, although the improvement in simulations of specific regions is equivocal. We predict a future global mean warming of 0.3 K per decade for greenhouse gases alone, or 0.2 K per decade with sulphate aerosol forcing included. By 2050, all land areas have warmed in our simulations, despite strong negative radiative forcing in some regions. These model results suggest that global warming could accelerate as greenhouse-gas forcing begins to dominate over sulphate aerosol forcing.

The general circulation model (GCM) used is the Hadley Centre climate model, a development from an earlier model⁵.

Modified formulations of the atmospheric dynamics⁶, convection⁷, land surface, boundary layer⁸ and cloud⁹ schemes have been used. The horizontal resolution is $2.5^\circ \times 3.75^\circ$ (latitude $\times$ longitude), with 20 layers in the ocean and 19 layers in the atmosphere. We apply calibrated seasonal flux adjustments to ocean surface temperatures and salinities⁵, to bring about a faithful representation of present mean climate. A simple parametrization of ice-drift¹⁰ is included, obviating the need for flux adjustments to sea ice. The equilibrium sensitivity to a doubling of CO_2 concentration is estimated to be 2.5 K, lower than most GCMs².

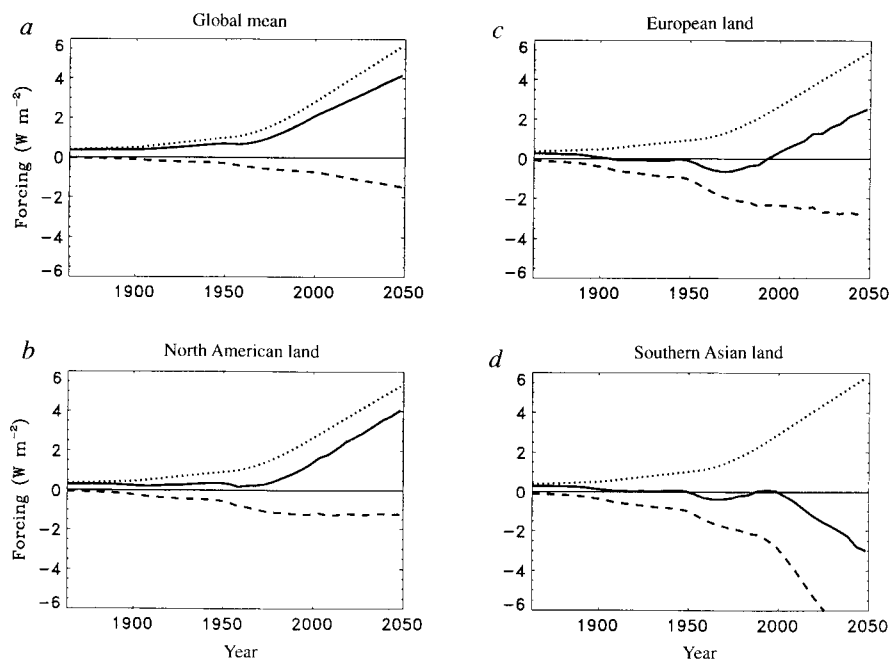
The model was brought to near equilibrium through a total of 470 years coupled simulation, after which the control simulation commenced. There is no detectable trend in global mean surface temperature in the 300 model years of the control run, although there is a slow warming of the deeper ocean layers amounting to a maximum of 0.07 K per century in the global mean at 1,500 m depth. The net heating of the total system is less than 0.2 W m^{-2} .

Three experiments were performed, each starting at model year 1860: a control with constant CO_2 concentrations, an experiment GHG in which the concentration of CO_2 was increased gradually to give the changes in forcing due to all greenhouse gases, both in the past and to 2050 under a given scenario, and an experiment SUL in which both greenhouse gases and the direct radiative effect of sulphate aerosols were represented. The concentrations of sulphate aerosols and greenhouse gases after 1990 were based on IPCC scenario IS92a² which assumes a slow reduction in the rate of economic growth and gradual increase in conservation measures (Fig. 1).

The greenhouse-gas forcing increases slowly from 0.4 W m^{-2} relative to the control in 1860 to 1.2 W m^{-2} in 1960, then more rapidly reaching 2.5 W m^{-2} in 1990, and thereafter at 0.6 W m^{-2} per decade (Fig. 1a). The global mean sulphate aerosol forcing increases continuously, and most rapidly, from 1950 to 1990 (Fig. 1a). Note that in recent decades, the percentage increase per year of emissions has grown faster than the global average

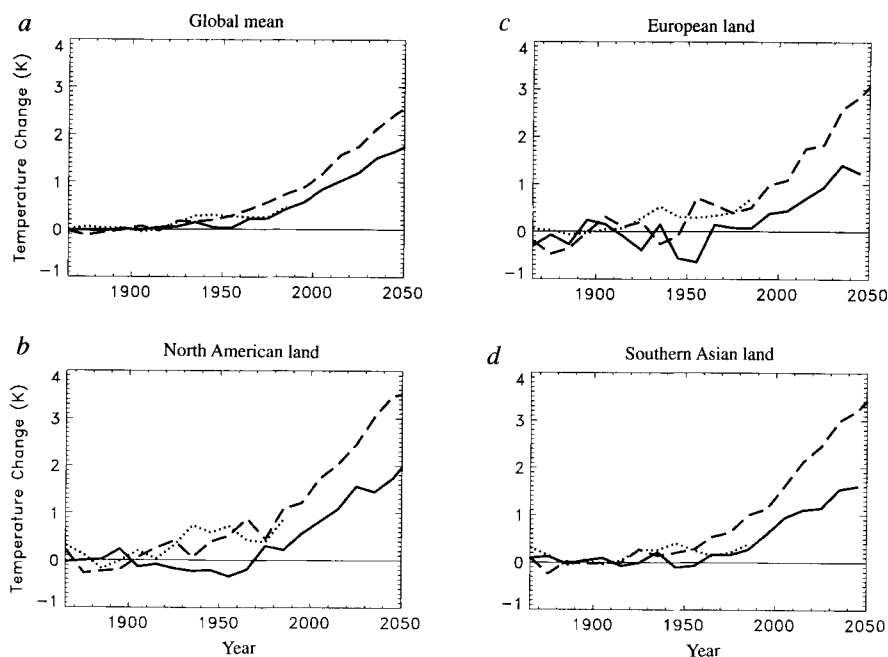
FIG. 1 Area-average annual mean radiative forcing owing to: increasing concentrations of greenhouse gases (experiment GHG, dotted curve); the direct effect of sulphate aerosols represented by increasing surface albedo (dashed curve); net forcing (experiment SUL, solid curve). a, The global mean; b, North America—land between 30° and 60°N and 40° to 140°W ; c, Europe—land between 35° and 70°N and 15°W to 60°E ; d, southern Asia—land between 7.5° and 42.5°N and 60° to 130°E .

METHODS. The concentration $C(t)$ of CO_2 at time t was chosen to give the estimated forcing (F) relative to the control (fixed concentration C_0) $F = 6.3 \ln(C(t)/C_0) \text{ W m}^{-2}$ due to increases in all greenhouse gases from 1765 (ref. 2, Table 2.6). After 1990, $C(t)$ was increased by $1\% \text{ yr}^{-1}$, which is within 0.2 W m^{-2} of IPCC scenario IS92a². There is an initial increment of 0.4 W m^{-2} at 1860 arising from changes in greenhouse gas concentration from 1765 to 1860. The direct effect of sulphate aerosols was added in experiment SUL by increasing the surface albedo in the clear-sky fraction of each grid box^{8,22}. The pattern of aerosol loading to 1990 was based on the calculated annual mean distribution for the 1980s²³, scaled by the estimated annual mean sulphate emissions^{24,25}. The global mean forcing of 0.6 W m^{-2} at 1990 lies within recent estimates of $0.3\text{--}0.9 \text{ W m}^{-2}$ (refs 18, 19). We have ignored seasonal variations of the pattern and the indirect effect of sulphate aerosols on cloud brightness⁹. A sulphate distribution for 2050 (H. Rodhe and U. Hansson, personal communication) was obtained



using a sulphur-cycle model²³ with the sulphur emissions of scenario IS92a. From 1990 to 2050, the loading pattern was interpolated between 1990 and 2050 values, with the field scaled to give the global loading of scenario IS92a.

FIG. 2 Changes in area-average decadal mean temperature at 1.5 m, relative to the 1880–1920 mean. Observed⁴ (dotted curve), GHG (dashed curve), SUL (solid curve). a, Global mean; b, mean over North America; c, mean over Europe; d, mean over southern Asia. Areas defined as in Fig. 1.



over southern Asia, and slower over western countries¹¹ where in the last decade there has been a decrease.

Reproduction of the past observational record within the limits of natural variability is a necessary, though not sufficient, condition for a model to produce reliable estimates of climate change. The differences in global mean temperature between simulations and observations⁴ are within the range of simulated internal variability until about 1940 (Fig. 2a). The model's variability is generally greater than observed on timescales up to several decades. (On longer timescales, the estimates of internal variability based on the observations will be exaggerated by any long-term trend due to external forcing.) In the 1940s and 1950s, SUL is significantly cooler than the observations at the 5% significance level (two-tailed test assuming that decadal means are normally distributed with the same standard deviation, 0.073 K, as the control simulation). There is a 14% probability that at least two of the decades from 1860 to 1990 would be significantly different at this level by chance. Alternatively, the prescribed forcing (Fig. 1a) may be incorrect. The response to the 1.6 W m^{-2} change in forcing by 1990 in SUL is only 0.4 K, suggesting that it would require an increase in forcing of the order of 1 W m^{-2} to produce the extra warming in the 1940s. This seems unlikely, even with the large uncertainties in forcing³. For example, the optical depth of volcanic aerosols may have been a minimum¹², but the forcing anomaly was probably less

than 0.5 W m^{-2} . A combination of natural fluctuations¹³ and small errors in forcing seems the most likely explanation.

From 1970, the difference between GHG and observations⁴ is significant in each decade at both the 5% (and 1%) levels whereas SUL is not significantly different from the observations at the 5% level after the 1930s and 1940s. Thus including the cooling attributed to sulphate aerosols (SUL) gives a simulation closer to the observations in recent decades, as found in energy-balance models^{14–16} or idealized GCMs¹⁷. Note the rapid warming after 1970 in the observations and in SUL. In SUL, this is the response to accelerated greenhouse warming and a slower rate of increase in cooling from sulphate aerosols (Fig. 1a).

Comparison of the simulated spatial distribution of changes provides a potentially more stringent test of the model's credibility. We show decadal results starting with the 1860s, but focus mainly on the changes in recent decades when the observed data coverage is better and the forcing and signal-to-noise in the GHG and SUL experiments is largest (Figs 1, 2). We first compute decadal anomalies for GHG, SUL and the observations in the same manner by subtracting the respective 1860–1990 means from each decadal mean. Then we compute a centred spatial correlation for each successive decade between the model experiments and the observations. For the decades since 1950, the magnitude of the pattern correlation between SUL and the observations increases steadily, rising above the 10% significance

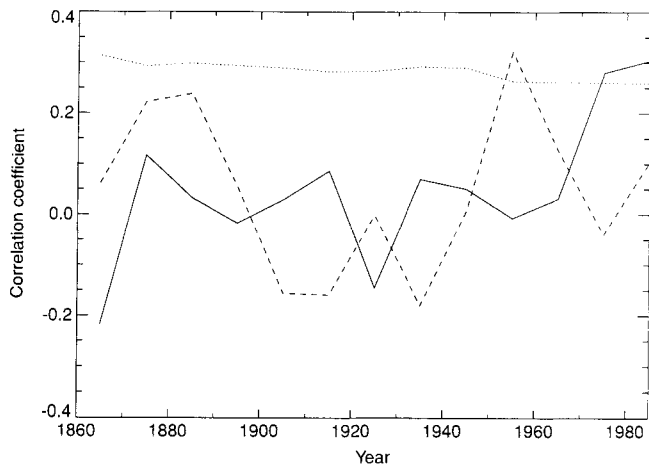


FIG. 3 Spatial correlation between simulated and observed decadal temperature changes relative to the 1860–1990 mean. Dashed line, GHG; solid line, SUL. The dotted line gives the 10% (one-tailed) level of significance, which varies with data coverage.

METHODS. Observed annual means were computed where there is at least one value in every month in a 5° grid-box. Observed and simulated data were averaged on a $15^\circ \times 15^\circ$ grid, and decadal means formed in grid-boxes containing at least one annual observed value. The significance level was estimated from the distribution of correlations between decadal means in the control, assumed to be gaussian, using the observational mask for each decade.

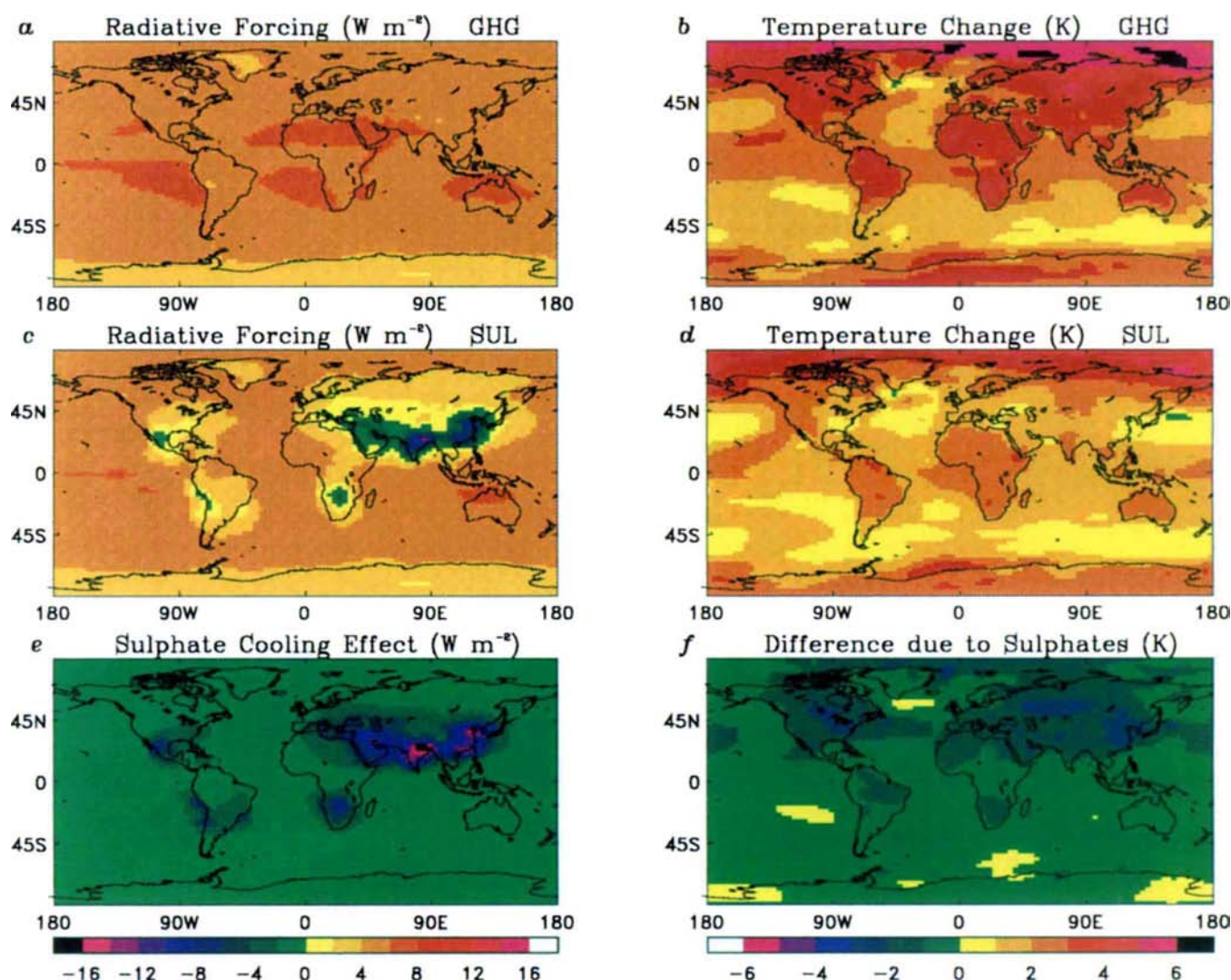


FIG. 4 Annual mean changes, averaged over 2030–50 relative to the control simulation. Temperature contours (b, d, f and right-hand scale bar) every 1 K, forcing contours (a, c, e and left-hand scale bar) every 2 W m^{-2} . a, b, Forcing (a) and temperature change (b) in GHG. c, d, Forcing (c) and temperature change (d) in SUL. e, f, Radiative cooling

(e) and difference in response (f) due to adding sulphate aerosols. The greenhouse-gas forcing at year t is obtained by scaling the change at the top of the troposphere on instantaneously doubling CO_2 by $\ln(C(t)/C_0)/\ln 2$. The sulphate aerosol forcing is evaluated at the top of the atmosphere timestep by timestep.

level in the two most recent decades (Fig. 3). This recent trend is consistent with what could also be an emerging greenhouse gas/sulphate aerosol signal in the observations. The GHG correlation in the decade beginning in 1950 rises above the 10% significance level, but subsequent values are much smaller despite increasing forcing in the more recent decades. Thus the spatial patterns of surface temperature anomalies in SUL, compared to GHG, more closely resemble those in the observations in recent decades when the forcing is largest.

The distinction between the simulations is less evident in specific regions because of the high level of variability of temperature on smaller scales. GHG is substantially warmer than observed over southern Asia after 1960 (Fig. 2d). However, SUL is generally cooler than observed over Europe and North America after the 1940s (Fig. 2b–d). Possible explanations include excessive sulphate aerosol forcing in these regions and natural variability.

After 1990, the rate of global warming in GHG increases to 0.3 K per decade (Fig. 2a). In SUL, the rate increases rapidly to 0.2 K per decade, as the net forcing increases (Fig. 1a). Southern Asia continues to warm despite increasingly negative forcing (Fig. 1d), although at less than half the rate in GHG

(Fig. 2d). Over North America and Europe, the aerosol forcing levels off and the greenhouse warming is more evident, though still less than in GHG. The increase in mean sulphate aerosol forcing still has a global effect, even where the local loading is substantially unchanged. The extension of these sensitivity studies to 2050 under a prescribed scenario indicates the potential strong influence of sulphate aerosols on regional climate change.

The patterns of forcing and response, averaged over 20 years from 2030, are shown in Fig. 4. The radiative forcing in GHG shows relatively little regional variation¹⁸ (Fig. 4a). The response is enhanced in high latitudes by sea-ice feedbacks, and slowed in the Southern Ocean and the northern North Atlantic by deep mixing in the ocean (Fig. 4b), as in other transient experiments⁵. In SUL, there are areas of both positive and negative forcing (Fig. 1), but by 2030–50, areas of substantial net negative forcing are restricted to southern Asia (Fig. 4c). Even so, this region remains warmer than in the control simulation, due to the movement of warmer air from surrounding areas (Fig. 4d). The largest decreases in temperature on adding aerosols occur in northern mid-latitudes, where the forcing is largest, and in the Arctic, where the global-scale cooling is amplified by increases

in sea ice^{8,19,20} (Fig. 4e,f). There is considerable variability on decadal and longer timescales—the standard deviation of 20-year means from the control simulation is 0.2–0.4 K over the extratropical continents—but the differences between GHG and SUL are several times larger than this and thus statistically significant. A more rigorous statistical assessment of the geographical distribution of changes will form a separate study.

We have shown that inclusion of sulphate aerosol forcing improves the simulation of global mean temperature over the last few decades, although further work is needed to clarify why the simulation over North America and Europe is not improved. There remain uncertainties in model sensitivity, particularly associated with clouds²¹, and in the external forcing due to sulphate and other aerosols, tropospheric ozone and solar variability³, and natural variability on long (greater than decadal) timescales. In particular, this study suggests that if we are to improve model predictions of climate change on global and regional scales it is not sufficient to consider greenhouse gases alone; the effects of aerosols and perhaps other forcing factors must be included. □

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Glacial climate instability in the Northeast Pacific Ocean

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RECENT climate records from Greenland ice cores^{1,2} and North Atlantic sediments^{3–5} have challenged the long-held notion that Pleistocene climate fluctuates between two relatively stable states (glacials and interglacials). It has been appreciated for some time that the transitions from one state to another are not smooth⁶, but the new records indicate that the glacial and interglacial periods themselves appear to be punctuated by significant climate variability—several short interstadial events punctuated the last glacial period, for example. But it has not been clear whether this climate instability is a global phenomenon or is peculiar to the North Atlantic region. Here we present climate proxy records from sediment cores from the eastern margin of the North Pacific Ocean, which indicate that climate in this region was also highly unstable during the last glaciation. Our observations suggest that glacial climate instability throughout the Northern Hemisphere might be linked to rapid changes in the size of the Laurentide ice sheet and associated changes in atmospheric circulation.

There is no clear consensus as to the origin of the observed climate instability that marks the last glacial period. Are the rapid shifts from one climate state to another the result of internal or external forcing? Broecker *et al.*^{7,8} were the first to suggest that the rapid climate changes observed in Greenland ice cores represent shifts between two climate modes and that these fluctuations were the result of turning on and off the North Atlantic thermohaline circulation. As an alternative to changes in the ocean–atmosphere system, a model has been proposed in which internal ice-sheet dynamics generated cyclical changes in the size of the Laurentide ice sheet and that these ice volume changes

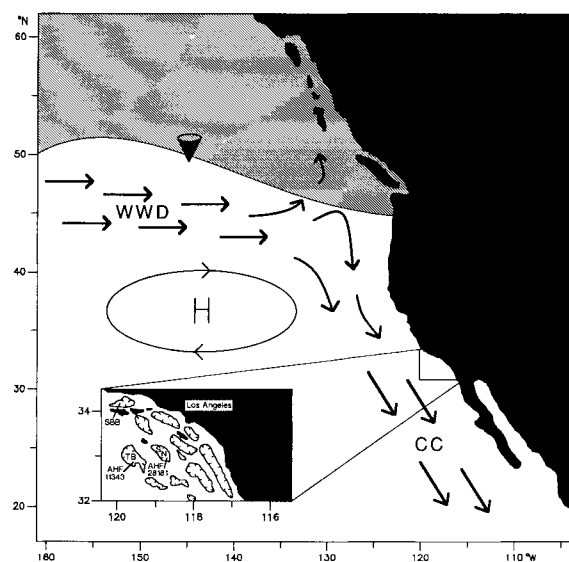


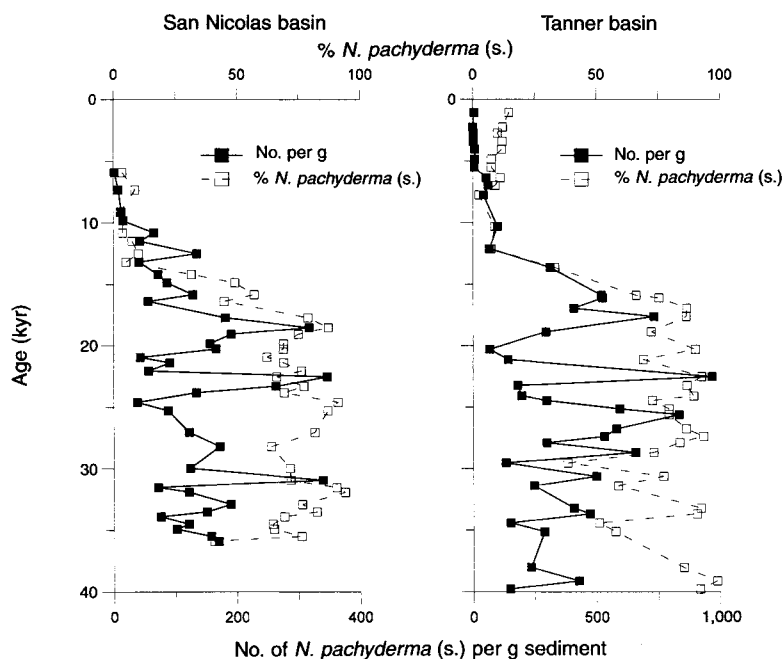
FIG. 1 Main figure, map of the northeast Pacific showing the location of the North Pacific high-pressure centre (H), and the major surface currents (CC, California Current; WWD, West Wind Drift). The lightly shaded area represents the present-day range of left-coiling *N. pachyderma* (based on Be¹¹). The southern boundary of this range is approximately the winter position of the 8 °C surface-water isotherm. The location of our previous subpolar North Pacific sediment-trap study¹² is indicated by the cone. Inset, the locations of the two California Borderlands cores used in this study (TB, Tanner basin; SN, San Nicolas basin).

caused the observed instability in North Atlantic climate during the last glacial^{9,10}.

We have conducted a detailed micropalaeontological study of two cores from the Tanner and San Nicolas basins of the California Borderlands region (Fig. 1) in order to evaluate climate variability in the northeast Pacific during the last glacial period. In particular, we use changes in the abundance of the planktonic

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FIG. 2 *N. pachyderma* records plotted as a function of age for the cores from San Nicolas basin (left) and Tanner basin (right). The solid squares (■) represent the actual number of left-coiling (sinistral, S.) specimens per gram of sediment, and the open squares (□) are the percentage of left-coiling specimens in the total *N. pachyderma* population.



foraminifera *Neogloboquadrina pachyderma* (sinistral) as a proxy for changing sea surface temperatures in this region during the past 35,000 years (sinistral indicates left-coiling). This species was selected because its distribution is strongly temperature controlled. In the modern ocean, this species is restricted to subpolar and polar regions¹¹ (Fig. 1). Sediment-trap studies from both the North Pacific¹² (Fig. 1) and the North Atlantic¹³ demonstrate that the production of left-coiling *N. pachyderma* is limited primarily to times of the year when sea surface temperatures are less than 8.0 °C. In addition, we have completed a number of sediment-trap studies in various parts of the Southern California Borderlands^{14,15} and find that the left-coiling variety of *N. pachyderma* is not found in the planktonic foraminiferal population now inhabiting this region. Based on these observations of the

modern ecology of *N. pachyderma* (sinistral), we interpret the presence of this species in the Borderlands at times in the past to be an indication of the southerly migration of cold subpolar waters into this region. We note that Coulbourn *et al.*¹⁶ found left-coiling *N. pachyderma* in low abundance in (recent) surface sediments from near Tanner basin, but we interpret this as probably the result of reworking of glacial material.

Age control for the two Borderlands cores used in this study is based on accelerator mass spectrometry (AMS) ¹⁴C dating of planktonic foraminifera (Table 1). The AMS ¹⁴C dates were corrected by subtracting 400 years from the initial radiocarbon results to account for the age difference in ¹⁴C between the atmosphere and surface waters at 30° N in the Pacific¹⁷. These ¹⁴C ages were then converted to calendar ages¹⁸ and age estimates for each sample were determined assuming constant sedimentation rates between ¹⁴C ages.

Time-series records of the absolute abundance (number of specimens per gram of sediment) of *N. pachyderma* (sinistral), and the percentage of total *N. pachyderma* specimens that are left-coiling, are plotted versus time for each core in Fig. 2. Two important features are evident in the records for both cores. First, the transition from the last glacial to the Holocene is marked by an abrupt change in the coiling dominance of the *N. pachyderma* population; the glacial period is dominated by left-coiling forms and the Holocene by right-coiling forms. A similar observation has been made in several previous studies of cores from this region^{19,20}. Coincident with this coiling change is a sharp decline in the absolute abundance of left-coiling *N. pachyderma*. During the Holocene, the left-coiling morphotype is virtually absent. These latter findings are consistent with our sediment-trap results which show that sinistral *N. pachyderma* does not populate this region at present^{14,15}.

The second striking feature of Fig. 2 is the high-frequency variability in the abundance of *N. pachyderma* (sinistral) during the last glacial period. The period from approximately 35 kyr to 15 kyr ago contains a series of abrupt increases and decreases in this species in both cores. The overall quality of foraminiferal preservation during the glacial interval is good²¹ and so the observed variability in abundance of *N. pachyderma* cannot be attributed to carbonate dissolution. Rather, we interpret this record as indicating rapid, large-amplitude changes in the flux of this species to the sea floor.

TABLE 1 Radiocarbon ages for sediment cores

Depth (cm)	¹⁴ C corrected age* (yr)	Calendar age† (yr)
Core AHF-11343		
105	6,930 ± 80	7,753
153	9,005 ± 80	10,326
205	13,490 ± 110	15,888
221	14,850 ± 160	17,574
256	15,600 ± 570	18,504
320	18,560 ± 300	22,174
368	21,330 ± 180	25,609
565	33,630 ± 650	40,861
Core AHF-28181		
55	8,470 ± 135	9,663
90	13,355 ± 150	15,714
120	16,600 ± 240	19,744
144	18,750 ± 210	22,410
164	20,850 ± 200	25,014
184	25,570 ± 320	30,867
234	29,565 ± 465	35,820

* Correction assumes a 400-year age difference between surface water carbon and atmosphere carbon at this location in the northeast Pacific¹⁷. Analyses were on mixed species of planktonic foraminifera and were performed at the University of Arizona.

† ¹⁴C ages were converted to calendar ages using the equation given by Bard *et al.*¹⁸.

The observed changes in coiling and abundance of *N. pachyderma* have important implications for climate and oceanographic conditions in the eastern North Pacific during the last glacial–interglacial cycle. We use the modern observed relationships between the production of *N. pachyderma* (sinistral) and surface water conditions (for example, sea surface temperature)^{12,13} as a basis for inferring either palaeotemperatures or the presence of different water masses in this region during the past. The fact that the *N. pachyderma* population in the Southern California Borderlands was dominated by left-coiling individuals during the last glacial suggests that subpolar-like waters migrated southwards along the eastern margin of the North Pacific to this latitude (33° N). Furthermore, the modern planktonic foraminiferal fauna that is most similar to this glacial assemblage is associated with winter sea surface temperatures of about 8 °C (refs 12, 13). If the same fauna–temperature relationship existed during the last glacial, it would indicate that sea surface temperatures in the Borderlands region were about 6 °C colder than present. A similar glacial–interglacial temperature change was proposed for this region based on oxygen-isotope results²⁰. This represents a significantly larger glacial–interglacial temperature difference than previously reported for this region²².

The rapid fluctuations in abundance of left-coiling *N. pachyderma* during the last glacial suggest that sea surface conditions in this area were highly unstable. Rather than being uniformly cold, there were periodic incursions of subpolar waters as far south as the Borderlands during the last glacial. We suggest that these rapid advances and retreats of subpolar waters were related to changes in atmospheric circulation and its concomitant impact on the California Current. According to the model simulations of the Cooperative Holocene Mapping Project²³ (COHMAP), the North Pacific high-pressure centre (the North Pacific High) was displaced to the south during the last glacial maximum. This shift may have strengthened the California Current, bringing subpolar surface waters, at least periodically, to the Borderlands region.

Oxygen-isotope records from Greenland ice cores reveal a number of large-scale fluctuations in air temperature (Dansgaard–Oeschger cycles) during the last glacial interval that have periods of 2–5 kyr (ref. 1), with age-equivalent changes in sea surface temperatures also occurring in the high latitudes of the North Atlantic⁴. This glacial climate instability is not as apparent in Antarctic ice-core records²⁴ and it has been postulated that these rapid ocean/atmosphere changes are somehow restricted to the North Atlantic region¹. Recent work suggests, however, that some of the interstadial events seen in the Greenland records are also present in the Antarctic (Vostok) cores²⁵.

Our results from the California Borderlands suggest that glacial climate instability is not a regional phenomenon found only in the North Atlantic. Indeed, continuing studies of Santa Barbara basin sediments²⁶ and western North American glacial

deposits²⁷ reveal a climate record similar to that seen in the North Atlantic region and thus corroborate our findings. The question to be answered is what is the linkage between the climate instability observed in the North Atlantic and the North Pacific. One possible link may be through changes in ice-sheet topography. Continental elevation plays an important role in controlling large-scale features of atmospheric circulation. Using a global atmospheric general circulation model, Broccoli and Manabe²⁸ found that the increased extent of continental ice during the last glacial produced more of a cooling in the Northern Hemisphere than in the Southern Hemisphere.

The flow of the upper-level westerlies or jet stream plays a dominant role in controlling low-level atmospheric circulation patterns in the Northern Hemisphere. The jet stream serves as a boundary between cold Arctic air-masses and warm tropical air-masses. In addition, the North Pacific high-pressure cell is centred beneath the jet stream. The latitudinal position of both the jet stream and the North Pacific High change seasonally in response to changes in the equator-to-pole temperature gradient. At present, the summer jet stream is positioned at approximately 50° N when it crosses the west coast of North America, with the North Pacific High being south of the jet stream (Fig. 1). In contrast, it has been suggested that the presence of a large ice sheet over much of North America during the last glacial resulted in a splitting of the jet stream, with the southern branch being stronger than the present day jet and positioned over the subtropical North Pacific and southern North America^{29,30}. Because the jet stream serves as a boundary between warm and cold air masses, its position during the last glacial would have resulted in significantly colder temperatures in the Borderlands region. In conjunction with the relocation of the jet stream, the North Pacific High also migrated to the south during the last glacial period²³. This may have strengthened the flow of the California Current and brought cold subpolar waters to more southerly latitudes at this time.

According to the 'binge–purge' model of MacAyeal⁹, the Laurentide ice sheet underwent repeated periods of growth and decay during the last glacial. At the culmination of each growth period (binge phase) the Laurentide ice sheet may have reached a thickness of over 3,000 m. This was followed by a period of rapid ice discharge (purge phase) during which ice thickness may have decreased⁹ by 1,250 m. The input of large volumes of ice into the North Atlantic may have caused a shutdown or reduction of North Atlantic Deep Water production and a cooling throughout this region^{4,9}. In addition, because the size of the Laurentide ice sheet has an effect on atmospheric circulation patterns^{23,31}, the binge–purge cycles may have resulted in repeated shifts in the latitudinal position of both the jet stream and the North Pacific High, producing rapid climate changes in this region. Thus, these cyclic changes in the size of the Laurentide ice sheet may serve as one possible way to link the unstable climate patterns observed in both the North Atlantic and North Pacific. □

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Two stages of visual processing for radial and circular motion

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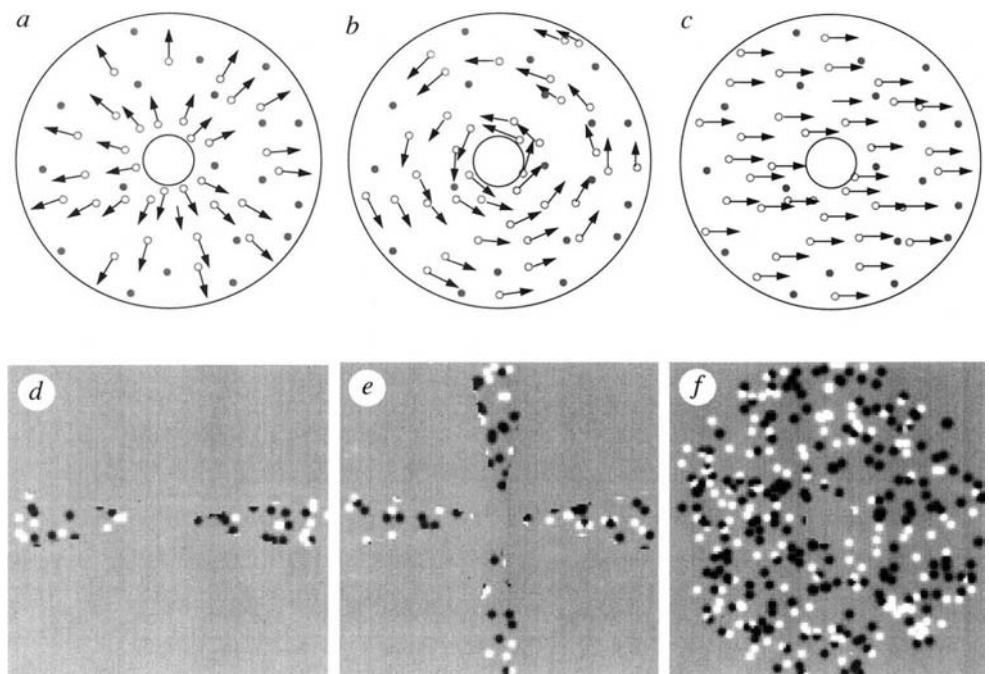
As we move through our environment, the flow of the deforming images on our retinae provides rich information about ego motion and about the three-dimensional structure of the external world. Flow-fields comprise five independent components, including radial and circular motion¹⁻³. Here we provide psychophysical evidence for the existence of neural mechanisms in human vision that integrate motion signals along these complex trajectories. Signal-to-noise sensitivity for discriminating the direction of radial, circular and translational motion increased predictably with the number of exposed sectors, implying the existence of specialized detectors that integrate motion signals of different directions from different locations. However, contrast sensitivity for complex motion did not increase greatly with sector number, implying that the specialized detectors are preceded by a first stage of local-motion mechanisms that impose a contrast threshold. These findings fit well with recent electrophysiological evidence in monkey⁴⁻⁷ showing that whereas motion-sensitive neurons in primary visual cortex respond best to

local translation, many neurons in the medial superior temporal cortex have large receptive fields tuned to radial, circular or spiral motion.

Observers viewed four-frame sequences of random dot displays, in which a proportion of dots were appropriately displaced between frames, the remainder being replaced at random. The dots fell within a 10° circle, notionally divided into 16 equal sectors. Signal dots could be confined to 1, 2, 4, 8 or all of the sectors, symmetrically arranged, whereas the remaining sectors were either left blank or filled with incoherently moving 'noise' dots of the same density (see Fig. 1). Sensitivity for motion discrimination was defined as the inverse of the minimum proportion of total dots in the signal sectors at which motion direction could be reliably discriminated ($1 + N/S$), where N is noise and S is signal. Figure 2 shows how motion sensitivity varied with stimulus area for radial, circular and translational motion. For all types of motion, sensitivity increased with stimulus area, implying that discrimination took advantage of motion signals from all sectors, summed within specialized neural mechanisms. For radial and circular motion, summation could not be based on detectors tuned solely to local direction, particularly when there were few sectors in the display: when there were only two sectors the motion in each sector was in opposite directions, and when there were four it was orthogonal. Note also that sensitivity was lower when the non-signal sectors were filled with noise than when they were empty. The additional noise from remote sectors reduces sensitivity, probably because it is integrated within a common detector unit. Interestingly, in these conditions the coherent motion did not seem to be confined to the signal sectors, but the whole display appeared to expand, rotate or slide. The dotted and dashed lines of Fig. 2 represent

FIG. 1 Examples of stimuli. *a-c*, Schematic illustrations of the three types of motion; *d-f*, photographs of single frames of the actual stimuli. The complete pattern (*f*) subtended 10° at the eye, and was filled with 360 small black and white gaussian patches of space constant 0.5' (excluding the central 1.5°). Four frames were presented, each for 80 ms, within a temporal gaussian window of $\sigma = 50$ ms. On each frame, a proportion of the dots was displaced by 2' to produce radial, rotational or translational motion (illustrated by the arrows in *a-c*), and the remainder of the dots replaced at random (dots without arrows). The pattern was progressively curtailed to 1, 2, 4 or 8 sectors, each subtending 22.5° and maximally separated from the others (e.g. *d, e*). For the 'noise' conditions, the empty sectors were filled with incoherent dots of the same density, so that the spatial pattern on each frame resembled *f*, but coherent motion was confined to specific sectors.

METHODS. Three performance measures were obtained: (1) Signal-to-noise sensitivity for motion direction, defined as the inverse of the minimal proportion of coherent dots in the signal sectors ($1 + N/S$) at which the direction of motion could be discriminated; (2) contrast sensitivity (the inverse of Michelson contrast at threshold) for motion discrimination; and (3) contrast sensitivity for pattern detection. Within a given session, subjects were required to discriminate motion direction (expansion from contraction, clockwise from anticlockwise rotation, leftward from rightward or upward from downward translation); or to discriminate the pattern from one in which the signal sectors were set to mean-luminance (detection thresholds). From trial to trial, dot proportion or stimulus contrast was determined by the adaptive QUEST



procedure²⁵. Thresholds were calculated by fitting a cumulative gaussian curve to the psychometric data, about 200 points for each condition. The stimuli were prepared in advance on a Silicon Graphics workstation. Given the random nature of the stimuli and the problem of 'wrap-around' (when the motion took dots outside their sector they were replaced at random), the nominal signal-to-noise ratio was not always precise. We therefore measured it by autocorrelation along the motion trajectory (divided by overall signal power), and reclassified the stimuli accordingly. The stimuli were displayed on a Barco calibrator monitor by VSG framestore under computer control, at a resolution of 128 × 128 pixels, 190 Hz frame rate and 25 cd m⁻² mean luminance.

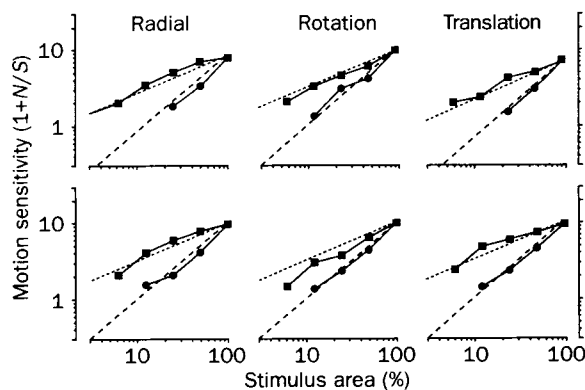


FIG. 2 Motion sensitivity for correctly discriminating the direction of radial, circular and translational motion (vertical for observer DCB, horizontal for observer MCM), as a function of stimulus area. Stimulus contrast was 0.5 (Michelson contrast). The squares refer to the condition where the non-signal sectors were set to average mean-luminance, and the circles to the condition where these sectors were filled with non-coherent dots of the same average density. For all three types of motion, sensitivity increased approximately linearly with stimulus area in the mask condition (dashed lines), and with the square root of stimulus area (dotted lines) in the no-mask condition. The dotted and dashed lines represent constant signal-to-noise ratios of an ideal integrator, that sums motion signals over the whole display, assuming the noise to be Poisson-distributed (so its variance is proportional to the number of noise dots). The results follow these predictions closely, implying the existence of specialized mechanisms that sum motion energy between sectors. Similar results were replicated in two other observers, one of whom was naive.

stimuli of constant signal-to-noise ratio, assuming linear integration^{8,9}. The prediction of an ideal integrator model is for a linear increase in sensitivity when the non-signal sectors were filled with noise dots, and a square-root increase when they were empty. In both conditions, the model provides an adequate fit to the data, indicating that the motion is discriminated by specialized operators of constant efficiency⁹.

We next measured summation of contrast sensitivity for these stimuli (only for radial motion). Here the dots in the signal sectors moved coherently, and the contrast of the whole display

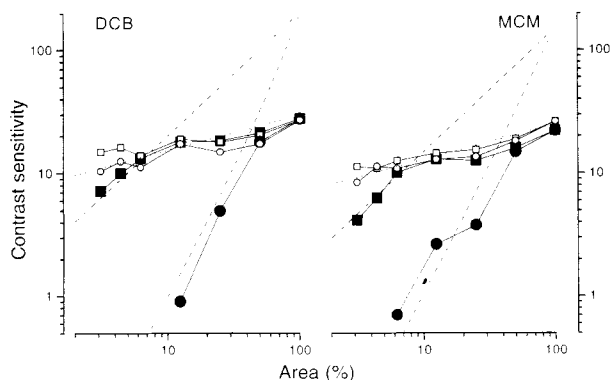


FIG. 3 Contrast sensitivity (Michelson contrast) for discriminating the direction of radial motion (filled symbols) and for detection of these patterns (open symbols). In this experiment, the three smallest stimuli had only one signal sector, varying in size from 10° to 22.5°. For all other stimulus areas, the sectors subtended 22° and were symmetrically opposed, as before. Squares refer to the condition when the non-signal sectors were set to average mean-luminance, and circles to when they were filled with noise dots of the same density and contrast. In the signal sectors $S/(S+N)=63\%$. The three curves show theoretical predictions for variation in sensitivity. The dotted line shows the

was adjusted to determine the threshold for discriminating the direction of motion, and for detecting the patterns (Fig. 3). When the non-signal sectors were filled with noise, there was strong summation, but little or no summation when they were empty. The dashed and dot-dashed lines show the predictions of the integrator model for two experimental conditions (see Fig. 3 legend). Contrast sensitivity data follow these predictions for only the smallest stimulus areas, particularly for the no-noise condition: for much of the range, sensitivity was far less than that predicted by the integrator, implying that other factors may limit performance. One possibility (consistent with the physiology) is that discrimination of complex motion involves two stages: local-motion detectors followed by integrator mechanisms tuned to complex motion. The dotted line through the open symbols of Fig. 3 shows how sensitivity should vary with area if it were limited by the contrast threshold of independent detectors responding locally to the direction of motion. The slight dependence on area results from the increased probability that a larger area will excite at least one local detector^{10,11}. The stimuli at all points along this line will elicit the same excitation in the integrator unit, so integration cannot enhance the limit imposed by the lower stages. Sensitivity for discrimination of complex motion should follow the predictions of the integrator model up to the point where it meets the limit set by the first-stage motion detectors. The data follow these predictions well, indicating that complex motion analysis involves two stages, local-motion detectors that limit stimulus visibility, and more specialized detectors that analyse expansion, rotation and translation (and combinations thereof) over extensive regions.

Several lines of psychophysical evidence are consistent with the existence of two stages of motion analysis. Contrast sensitivity for spatially curtailed sinusoidal gratings shows full summation over a relatively limited area (equal to about one cycle of periodicity of the stimulus^{12,13}), suggesting that performance for these stimuli is limited by first-stage contrast-dependent detectors, whereas the area of integration for direction discrimination (of translation) of limited-life random-dot patterns can be much larger, extending up to 9° (refs 14, 15) (a value that we have replicated informally in this study for all three types of motion). Morgan¹⁶ has shown that $D_{\max}$ (the largest dot-displacement to support coherent motion¹⁷) varies with dot size and stimulus blur in a way that suggests an early pre-filtering stage followed by a broad-band detector, an idea that has received recent sup-

prediction if detection and discrimination were limited by the action of local independent first-stage motion detectors. The slight increase in sensitivity with area results from 'probability summation' between the detectors¹⁰, given by $S_c = kA^{1/\beta}$, where S_c is contrast sensitivity, k is an arbitrary constant adjusted to achieve the best fit of the detection data, A is stimulus area and β the slope of the psychometric function, chosen as 3.5, on the basis of the present data and previous studies¹¹. The dashed and dot-dashed curves show the prediction of threshold sensitivity for a perfect integrator limited only by its internal noise N_i , given for constant $d'_e = 1$ (by definition at threshold)⁸, given by $d'_e = 1 = S_e / N_e = \alpha c D_s / \sqrt{N_i^2 + \alpha c D_n}$, where S_e and N_e are the signal and noise of the detector, D_s and D_n are the number of signal and noise dots, c is contrast and α is a constant governing contrast gain (of pre-integrator units). The last equality relies on the assumptions that the integration is quasi-linear at threshold, and that the noise in the stimulus is Poisson-distributed. Hence the integrated signal will be proportional to the product of contrast and dot number, and the total noise will be given by the square root of the sum of the internal and external noise variance. α and N_i were determined to give the best fit of the discrimination data, where the predicted sensitivity was less than that predicted from probability summation; otherwise the equation for probability summation was used for the fit. α was determined by the Simplex minimization procedure²⁶ to give the best fit of discrimination sensitivity in the noise condition (where N_i has negligible effect). N_i was then adjusted to fit simultaneously the data of both noise and no-noise conditions. The estimates of α and N_i (respectively) were 0.65, 0.3 for DCB and 0.7, 0.7 for MCM. See text for implications of the data.

port from masking studies¹⁸. Our results further suggest that the pre-filtering stage is directionally selective. Early experiments^{19,20} on adaptation suggested that radial and translational motion may be analysed by different mechanisms^{19,20}, although the quantitative predictions of the size of the receptive fields for these mechanisms (less than 1.5°) agrees neither with the results reported here nor with the physiology⁴⁻⁷: perhaps first-stage mechanisms may have mediated the adaptation to some extent. Despite these differences, however, the adaptation studies also suggested that radial motion may be a second-stage analysis, after a common contrast-dependent first stage²¹.

Modern electrophysiological studies have shown that motion is processed at several different cortical levels, including primary visual cortex (V1), middle temporal (MT) and medial superior temporal (MST) cortex. Neurons in V1 are selective to translational motion, and have relatively small receptive fields. In MT, receptive fields are larger, but the neurons remain selective to translational motion²². In the dorsal segment of MST (MSTd), cells have very large receptive fields, and many are selective to radial or circular motion⁴⁻⁷. However, selectivity is not restricted to these 'cardinal directions' of optic flow, as there also exist neurons selective to combinations of radial and circular motion (spiral motion) and to combinations of spiral and translational motion⁵⁻⁷. The experiments reported here do not address the issue of whether selectivity is restricted to the cardinal directions, but do show that there must exist neurons that respond to radial or circular motion trajectories. Other properties of MSTd neurons, such as their insensitivity to density, texture and relative depth of the texture elements^{4,5}, and particularly to the position

of the centre of the radial, circular or spiral motion^{6,7} (also supported by psychophysical evidence (R. J. Snowden and A. B. Milne, manuscript submitted)) make these neurons well suited for optic-flow field analysis, and for guiding the heading of ego motion^{23,24}. □

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Collapsin-induced growth cone collapse mediated by an intracellular protein related to UNC-33

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COLLAPSPIN¹, a member of the newly recognized semaphorin family²⁻⁴, contributes to axonal pathfinding during neural development by inhibiting growth cone extension¹⁻⁵. The mechanism of collapsin action is poorly understood. Here we use a *Xenopus laevis* oocyte expression system to identify molecules involved in collapsin signalling, because several experiments have raised the possibility that heterotrimeric GTP-binding proteins might participate in these events⁶⁻⁹. A collapsin response mediator protein of relative molecular mass (M_r) 62K (CRMP-62) required for collapsin-induced inward currents in *X. laevis* oocytes is isolated. CRMP-62 shares homology with UNC-33, a nematode neuronal protein required for appropriately directed axonal extension¹⁰⁻¹². CRMP-62 is localized exclusively in the developing chick nervous system. Introduction of anti-CRMP-62 antibodies into dorsal root ganglion neurons blocks collapsin-induced growth cone collapse. CRMP-62 appears to be an intracellular component of a signalling cascade initiated by an unidentified transmembrane collapsin-binding protein.

To characterize components of the collapsin response pathway, we injected RNA synthesized from a chick E7 dorsal root ganglion (DRG) complementary DNA library into oocytes, and then assessed the current response of voltage-clamped oocytes to bath application of collapsin. With collapsin-containing chick E10 brain membrane extracts (BME) (refs 6, 13) as ligand, a cDNA clone capable of rendering oocytes responsive was identified (not shown). This clone consists of 300 bp of 5' untranslated sequence followed by 890 bp of open reading frame (ORF) and no stop codon. We obtained a 3' extension of this clone by polymerase chain reaction and ligated the two fragments together to create CRMP-62. To determine if collapsin is the active component of BME in this assay, we expressed collapsin in COS cells and insect cells. Purified recombinant collapsin from baculovirus-infected cells has a specific activity of 10^6 U mg⁻¹ protein and produces pertussis toxin (PTX)-sensitive DRG growth cone collapse (Fig. 1a). The inward current in CRMP-62 RNA-injected oocytes initiated by BME (not shown), collapsin-expressing COS cell medium (Fig. 1b), and pure collapsin (not shown) are indistinguishable.

The CRMP-62 open reading frame encodes a protein of M_r 62K that has no signal peptide or membrane-spanning domain (Fig. 1c). The structure of CRMP-62 suggests that it is an intracellular protein, and that it may be required for coupling a transmembrane collapsin-binding receptor to a signalling cascade. Therefore naive oocytes must have low levels of an endogenous collapsin receptor that generates a current response only in the presence of CRMP-62. Crude DRG messenger RNA injection yields a larger response than can be accounted for by CRMP-62 alone, suggesting that DRG mRNA may encode both a collapsin-binding receptor and CRMP-62.

CRMP-62 is a novel sequence, sharing homology with three groups of sequences. The highest level of similarity is to several human fetal brain expressed sequence tags¹⁴. There are two human cDNAs, which we term *hCRMP-1* and *hCRMP-2*, sharing 84% and 98% predicted amino-acid identity with CRMP-62 (Fig. 1, and not shown). CRMP-62 is also related to *unc-33* (ref.

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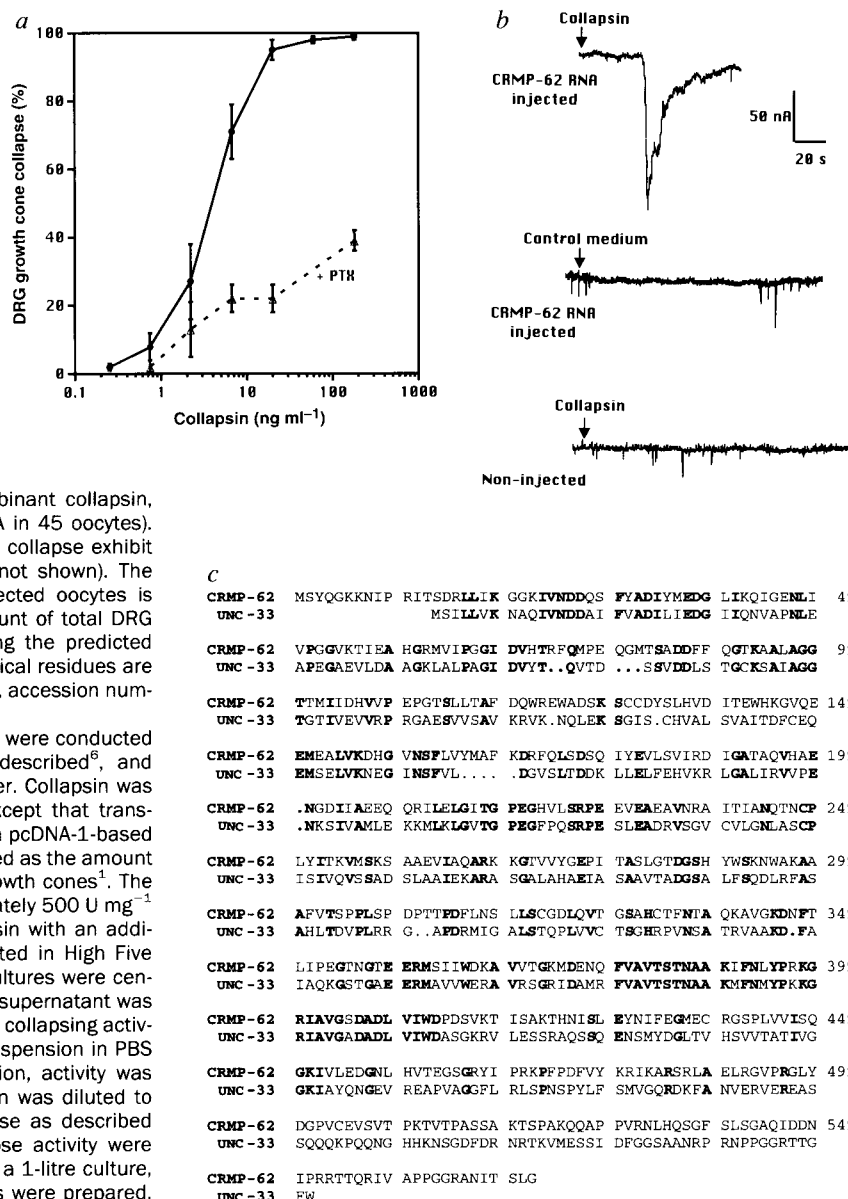
10), with 38% identity and 61% similarity (Fig. 1c). Without *unc-33*, nematode movements are uncoordinated and axon outgrowth is aberrant^{11,12}, reminiscent of the disordered outgrowth of insect neurons with perturbations of semaphorin expression^{2,5}. Although *CRMP-62* has similarity to bacterial D-hydantoinase proteins^{15,16}, purified recombinant *CRMP-62* has no detectable hydantoinase activity (<5 nmol hydantoin hydrolysed per min at 37 °C per mg protein).

We sought to determine whether *CRMP-62* reconstitutes a collapsin→G protein→phospholipase C→InsP₃→Ca²⁺→chlor-

ide channel cascade¹⁷. The reversal potential for BME-induced currents is -21 to -25 mV, identical to that for chloride (not shown). BME responses are abolished by prior injection of EGTA into oocytes (not shown), as expected for a Ca²⁺-mediated event. Furthermore, PTX decreases collapsin-induced currents (Fig. 2a). We also considered whether *CRMP-62* RNA injection might enhance G protein-coupled transduction generally, downstream of G protein activation. However, currents induced by lysophosphatidic acid (LPA) (Fig. 2b) and 5HT (not shown) are identical in non-injected and *CRMP-62* RNA-

FIG. 1 a, Collapse of DRG growth cones by purified collapsin is inhibited by PTX. Explants of E7 DRG were pretreated for 2 h with 500 ng ml⁻¹ PTX (broken line) or no additions (solid line). Different concentrations of purified collapsin were added and the fraction of collapsed growth cones was determined after 30 min incubation. For concentrations of collapsin greater than 10 ng ml⁻¹, PTX reduced growth cone collapse significantly ($P \leq 0.01$, Student's two-tailed t test). The data are the mean $\pm$ s.e.m. for 6 determinations. b, Collapsin induces an inward current in oocytes injected with *CRMP-62* RNA. Oocytes injected with *CRMP-62* RNA or not injected were voltage clamped after incubation for 2 days. Medium from COS cells transfected with pcDNA-collapsin was added at the time indicated by the arrow. An inward current is apparent in *CRMP-62* RNA-injected oocytes but not in control oocytes. Medium from COS cells transfected with pcDNA-1 did not alter membrane conductance. Non-injected control oocytes show no response to purified recombinant collapsin, even at concentrations as high as 200 U ml⁻¹ (>3 nA in 45 oocytes). Oocyte current responses and chick DRG growth cone collapse exhibit a similar dose response for recombinant collapsin (not shown). The amplitude of current response in *CRMP-62* RNA-injected oocytes is similar to that in oocytes injected with an equal amount of total DRG poly(A)⁺ RNA (not shown). c, An alignment comparing the predicted amino-acid sequences of *CRMP-62* and *UNC-33*. Identical residues are shown in bold. The *UNC-33* sequence is from GenBank, accession number Q01630 (ref. 10).

METHODS. Chick E7 DRG growth cone collapse assays were conducted as described⁶. Chick E10 BME was prepared as described⁶, and dialysed extensively against the oocyte perfusion buffer. Collapsin was expressed in COS cells essentially as described¹, except that transfected cells were cultured in serum-free medium and a pcDNA-1-based vector was used. One unit of collapsing activity is defined as the amount required in a 1-ml culture to collapse 50% of DRG growth cones¹. The specific activity of this COS cell medium was approximately 500 U mg⁻¹ protein. A recombinant baculovirus expressing collapsin with an additional six carboxy-terminal His residues was propagated in High Five *Spodoptera frugiperda* cells. After 68 h of infection, cultures were centrifuged at 1,000g for 10 min, and then the low-speed supernatant was centrifuged at 150,000g for 1 h. Nearly all growth cone collapsing activity was present in the high-speed pellet, but after resuspension in PBS plus 1 M NaCl and a second high-speed centrifugation, activity was released into the supernatant. The extracted collapsin was diluted to 150 mM NaCl and chromatographed over S-Sepharose as described for brain extracts¹. Those fractions containing collapse activity were more than 95% pure by SDS-PAGE (not shown). From a 1-litre culture, 400 to 800 µg protein was obtained. *X. laevis* oocytes were prepared, injected and voltage clamped as described¹⁸. Then, 2–4 days after RNA injection, collapsin (1–10 U ml⁻¹) was applied by mixing with the bath medium. Capped RNA was transcribed from a chick E7 DRG pcDNA-1-based cDNA library with T7 RNA polymerase and m7G(5')ppp(5')G. With BME as a ligand, 20 pools of 2,000 recombinants were screened to isolate clone 71. Because clone 71 was truncated at the 3' end, we used chick E7 DRG-derived cDNA as a template for PCR with oligo(dT) and a sense oligonucleotide 150 bp from the 3' end of clone 71. The major reaction product of 1.4 kb contained 150 bp of clone 71, then a NotI site, 810 bp of open reading frame, a stop codon, polyadenylation signal and a polyadenylate tract. Clone 71 was truncated because NotI was used during library construction. To construct pCRMP-62, clone 71 and the PCR-derived 3' fragment were ligated together at this NotI site. Databank searches revealed 8 human ESTs with homology to *CRMP-62*



(GenBank T06278, T08139, T08129, T09404, T06728, T07524, T05711 and M85617)¹⁴. We obtained plasmid clones for three of these from ATCC, and determined their nucleotide sequence. GenBank T06278 (ATCC 82946), contains a 1.9-kb insert with a 1.6-kb open reading frame, which we termed *hCRMP-1*. Five of the ESTs (T06278, T08139, T08129, T09404 and T06728) are identical to portions of this *hCRMP-1* sequence. The overlapped combination of open reading frames from ATCC 84159 and ATCC 82402 is the sequence we term *hCRMP-2*. Nucleotide sequences of double-stranded DNA were determined by the dideoxy chain termination method. The GenBank accession numbers for *CRMP-62*, *hCRMP-1* and *hCRMP-2* are U17277, U17278 and U17279, respectively.

injected oocytes. CRMP-62 protein expressed 2 days after RNA injection might participate directly in collapsin signalling, or might chronically alter the transcription of other proteins. To distinguish these hypotheses, we purified recombinant CRMP-62 (Fig. 2c) and then injected 10–30 ng CRMP-62 protein into each oocyte to yield a concentration of CRMP-62 at or below physiologic levels (see below). Then, 5 minutes after CRMP-62 protein injection, when transcriptional effects are likely to be negligible, collapsin elicits a transient inward current similar to that observed after RNA injection (Fig. 2d).

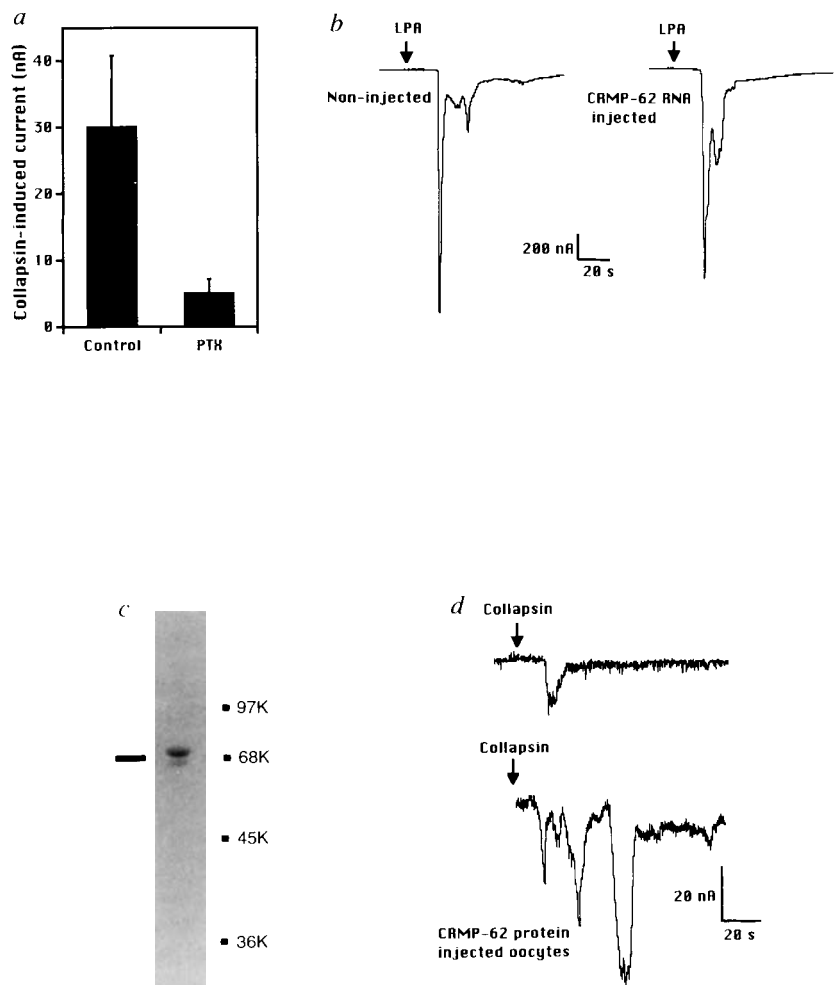
On northern blots, *CRMP-62* cDNA probe hybridizes to a 5.0 kb mRNA species in brain and DRG, but not other tissues (Fig. 3A). The expression of brain *CRMP-62* mRNA is highest at E7 and decreases by 95% in adulthood. *In situ* hybridization of E7 embryos detects high mRNA levels in the brain, retina, spinal cord and DRG (Fig. 3B). Brain levels are low in the immediate subventricular zone where primitive neuroblasts are dividing, but higher in adjacent neuronal layers of the telencephalon. Less signal is present in the developing molecular layer at the pial surface. The highest retinal levels are seen in the ganglion cell layer. Because retinal growth cones do not respond to collapsin¹, CRMP-62 function may not be restricted to collapsin.

CRMP-62 protein expression was characterized with antisera directed against residues 30 to 48 or 475 to 491 of CRMP-62. Immunoblot analysis of chick E7 tissues with anti-CRMP-62(30–48) shows high levels of immunoreactivity of 62K protein in the embryonic brain and DRG (Fig. 3C), and much lower levels of immunoreactivity at 58K. Similarly, anti-CRMP-62(475–491) recognizes a 62K protein in chick embryonic DRG (Fig. 3D). In brain, trace bands between 66 and 75K are also detectable with anti-CRMP-62(30–48). Immunohistologically, CRMP-62 is detectable at DRG growth cones (Fig. 3E). CRMP-62 is abundant, comprising 0.05% of embryonic brain protein (not shown). After maximal injections of 50 ng *CRMP-62* RNA, oocytes express slightly less CRMP-62 immunoreactivity than does embryonic chick brain (not shown). Non-injected oocytes do not express detectable CRMP-62. CRMP-62 immunoreactivity is found predominantly in cytosolic fractions of embryonic brain, but 20% of immunoreactive protein remains associated with particulate fractions during extensive washing at high ionic strength and can be extracted with detergent (not shown).

To assess the role of CRMP-62 in growth cone collapse, we used anti-CRMP-62(30–48) to block CRMP-62 function. Five minutes after injection into *CRMP-62* RNA-injected oocytes,

FIG. 2 CRMP-62 action in oocytes is PTX-sensitive, collapsin-specific and immediate. *a*, The effect of PTX pretreatment on the current response to recombinant collapsin in *CRMP-62* RNA-injected oocytes. PTX ($2 \mu\text{g ml}^{-1}$) was added to the incubation medium 24 h before the voltage-clamp experiment. The data shown are mean inward current $\pm$ s.e.m. for eight oocytes from the same frog in each experimental group. *b*, Oocyte response to lysophosphatides is not altered by injection of *CRMP-62* RNA. The inward current response after bath application of 100 nM LPA^{19,20} in a non-injected and *CRMP-62* RNA-injected oocyte is illustrated. The average peak inward current with and without injection of 40 ng *CRMP-62* RNA was 970 ± 350 nA and 780 ± 250 nA, respectively ($n = 7$). *c*, A recombinant CRMP-62 fusion protein was purified from *Escherichia coli* and analysed by SDS-PAGE. The position of *M*_r markers are shown on the right. *d*, Collapsin induces an electrophysiologic response in oocytes injected with recombinant CRMP-62 protein. 5 min after the injection of CRMP-62 protein, recombinant COS cell-derived collapsin was applied to the bath. Collapsin induced 20–50 nA inward currents in these two CRMP-62 protein-injected oocytes. This response is representative of 16 separate oocytes with a mean peak amplitude of 12.5 ± 3.6 nA. Buffer-injected oocytes showed no detectable response (< 2 nA) to collapsin ($n = 16$). CRMP-62 protein injection alone has no detectable effect on the holding current (not shown).

METHODS. Electrophysiologic procedures are described in Fig. 1. CRMP-62 (20 nl of 0.3 mg ml^{-1}) in 100 mM KCl, 10 mM Na, HEPES 0.1 mM DTT, pH 7.8 was injected into each 1,000-nl oocyte¹⁸. A bacterial expression vector encoding a fusion protein of *M*_r 68K with 6 His residues followed by the CRMP-62 sequence was created in pTrcHis (Invitrogen). Recombinant protein was purified on a nickel-containing resin under denaturing conditions from IPTG-induced *E. coli* carrying this plasmid. Full-length CRMP-62 fusion protein was further purified by SDS-PAGE, electroelution, dialysis into 8 M urea without SDS, and then dialysis into oocyte injection buffer (10 mM NaHEPES, 100 mM KCl, 0.1 mM DTT, pH 7.8). During the extensive dialysis to remove denaturing agents, the pH was maintained above 7.7 to avoid protein aggregation.



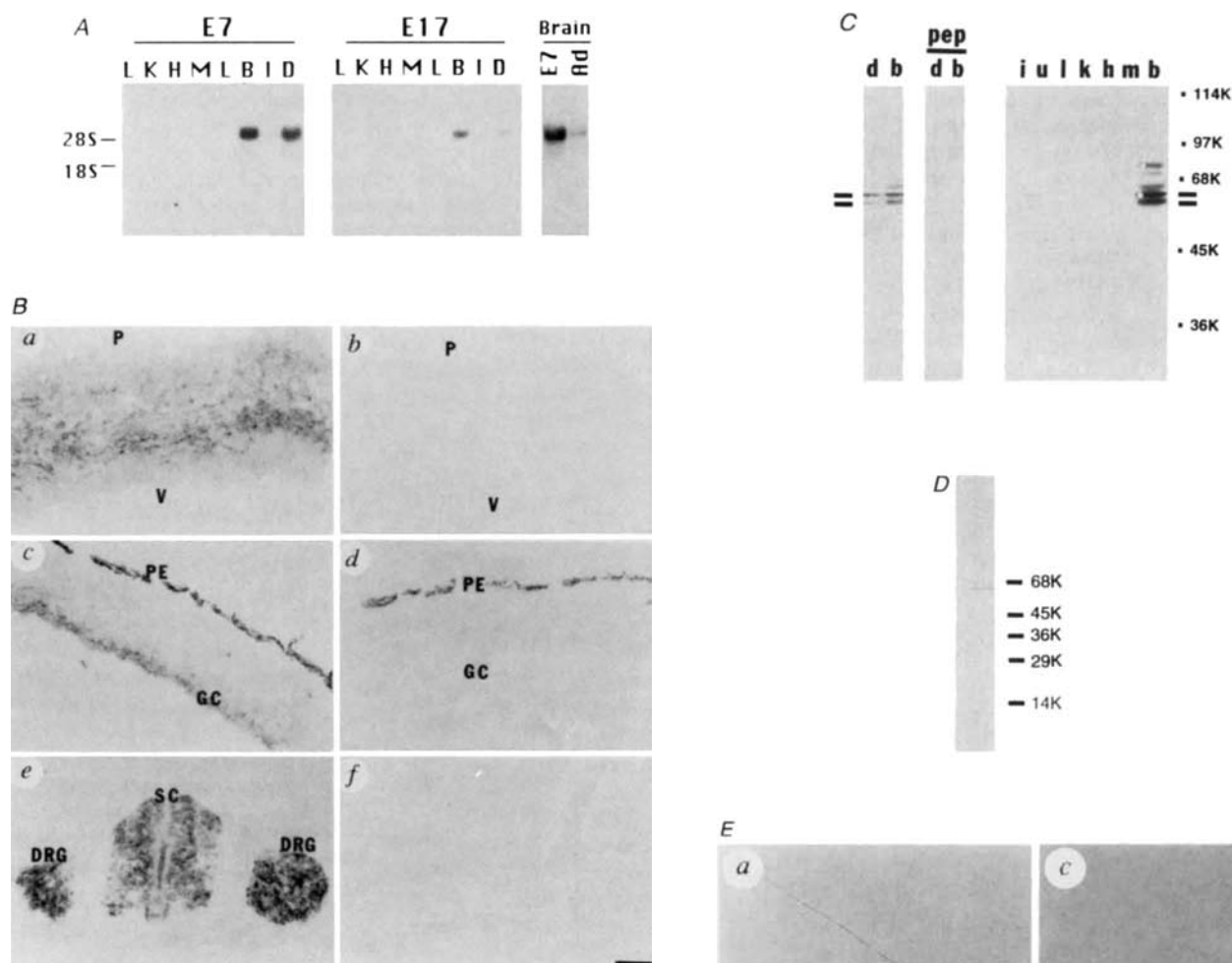


FIG. 3 Localization of CRMP-62 in developing neurons. **A**, Northern blot analysis of CRMP-62 expression. Total RNA (10 µg) from chick E7 or E17 embryonic tissues (from left: L, lung; K, kidney; H, heart; M, muscle; L, liver; B, brain; I, intestine; D, DRG) or adult (Ad) brain were hybridized to a CRMP-62 probe. The quantity of 18S and 28S ribosomal RNA was the same in each lane, and the migration of these RNAs is indicated at the left. **B**, Localization of CRMP-62 RNA in E7 chick embryo. E7 chick tissues were hybridized with an antisense (a, c, e, f) or sense (b, d) CRMP-62 probe. In the brain (a, b), CRMP-62 RNA is detected at high concentrations and is localized to deep layers of the telencephalon, but not in the region immediately adjacent to the cerebral ventricle (V). Lower levels are found in layers near the pial (P) surface. In the retina (c, d), CRMP-62 is highly expressed in the retinal ganglion cells layer (GC). The position of the pigment epithelium (PE) is indicated. In the lumbar region (e), there are high levels of CRMP-62 in the spinal cord (SC) and the dorsal root ganglia (DRG). The liver (f) and other organs (not shown) had no detectable CRMP-62 RNA. There is no staining with the sense probe (b, d). Scale bar, 60 µm (a-d, f), or 120 µm (e). **C**, Immunoblot analysis of CRMP-62(30-48) immunoreactivity in chick embryonic tissues. Immunoblots of total tissue homogenates containing 10 µg protein were prepared using 0.1 µg ml⁻¹ anti-CRMP-62(30-48). The DRG (d) and brain (b) show intense immunostaining, whereas other tissues (i, intestine; u, lung; l, liver; k, kidney; h, heart; m, muscle) show very little staining. The most prominent staining is at 62K, with lesser staining at 58K (indicated by the two lines at right and left). Fainter staining is observed in brain at higher M_r. The middle panel illustrates the absence of staining in the presence of the CRMP-62 peptide antigen (pep). The mobilities of markers are indicated on the right. **D**, CRMP-62(475-491) immunoreactivity in chick E7 DRG. The anti-CRMP-62(475-491) antibody at 1 µg ml⁻¹ exhibits a pattern of immunoreactivity for embryonic DRG tissue similar to that in C. The mobilities of markers are shown on the right. **E**, Localization of CRMP-62 in DRG growth cone. DRG explants were fixed 24 h after plating, and immunostained with anti-CRMP-62(30-48) antibody. Note the presence of CRMP-62 immunoreactivity in neurites and growth cones (a, b). There is no immunostaining in the presence of the 100 µg ml⁻¹ CRMP-62(30-48) MAP peptide (c shows absence of staining, d shows a differential interference contrast image of the same field to illustrate the presence of growth cones). Scale bar, 20 µm. **METHODS.** Total RNA was isolated from various tissues with acid guanidinium thiocyanate-phenol-chloroform, fractionated on 1.2% agarose-formaldehyde gels and transferred to a nylon membrane. The CRMP-62 probe was synthesized with random primed Klenow enzyme using the -88 to +633 BamHI fragment of CRMP-62 and [α -³²P]dCTP. After hybridization, the filter was washed to a final stringency of 0.1 × SSC and 0.1% SDS at 63 °C. Frozen sections (20 µm) of chick embryos

were treated sequentially with 3.7% formaldehyde, proteinase K, H₂O₂, formaldehyde, triethanolamine and acetic anhydride. Sense and antisense digoxigenin-labelled riboprobes were prepared from clone 71 plasmid DNA. Probes (3 µg ml⁻¹) were hybridized to sections in 50% formamide, 5 × SSC, 50 µg ml⁻¹ yeast transfer RNA, 1% SDS and 50 µg ml⁻¹ heparin at 60 °C. After RNase A treatment, sections washed with 50% formamide, 2 × SSC at 65 °C. Bound probe was detected using anti-digoxigenin antibody conjugated to alkaline phosphatase (Boehringer-Mannheim). Peptides corresponding to residues 30-48 (SFYADIYMEDGLIKQIGEN) and 475-491 (DFVYKRIKARSRLAELR) of CRMP-62 were synthesized on a branched lysine carrier core (MAP peptide, Research Genetics). Rabbits were immunized with MAP peptides and antibodies were affinity purified using an Affigel 15 resin containing 5 mg MAP peptide per ml resin. Both purified antibodies detect immobilized native recombinant CRMP-62 by ELISA at antibody concentrations above 25 ng ml⁻¹ (not shown). At 10 µg ml⁻¹, both antibodies immunoprecipitate native recombinant CRMP-62 stoichiometrically (not shown). Various tissues were homogenized in 20 volumes of 10 mM Tris-HCl, 1 mM EDTA, 1 mM PMSF, 5 µg ml⁻¹ leupeptin, 1 µg ml⁻¹ pepstatin A, pH 7.8, and analysed by immunoblot with anti-CRMP-62 and peroxidase-conjugated goat anti-rabbit IgG. In blocking experiments, the CRMP-62 MAP peptide (10 µg ml⁻¹) or recombinant CRMP-62 protein (100 µg ml⁻¹) was added to the primary antibody solution (0.1 µg ml⁻¹) before incubation with the blot. DRG explants were cultured as in Fig. 1 for 24 h, fixed with 3.7% formaldehyde, PBS, and then incubated with 1 µg ml⁻¹ anti-CRMP-62 antibody. Bound antibody was detected using peroxidase-conjugated goat anti-rabbit IgG with diaminobenzidine as substrate.

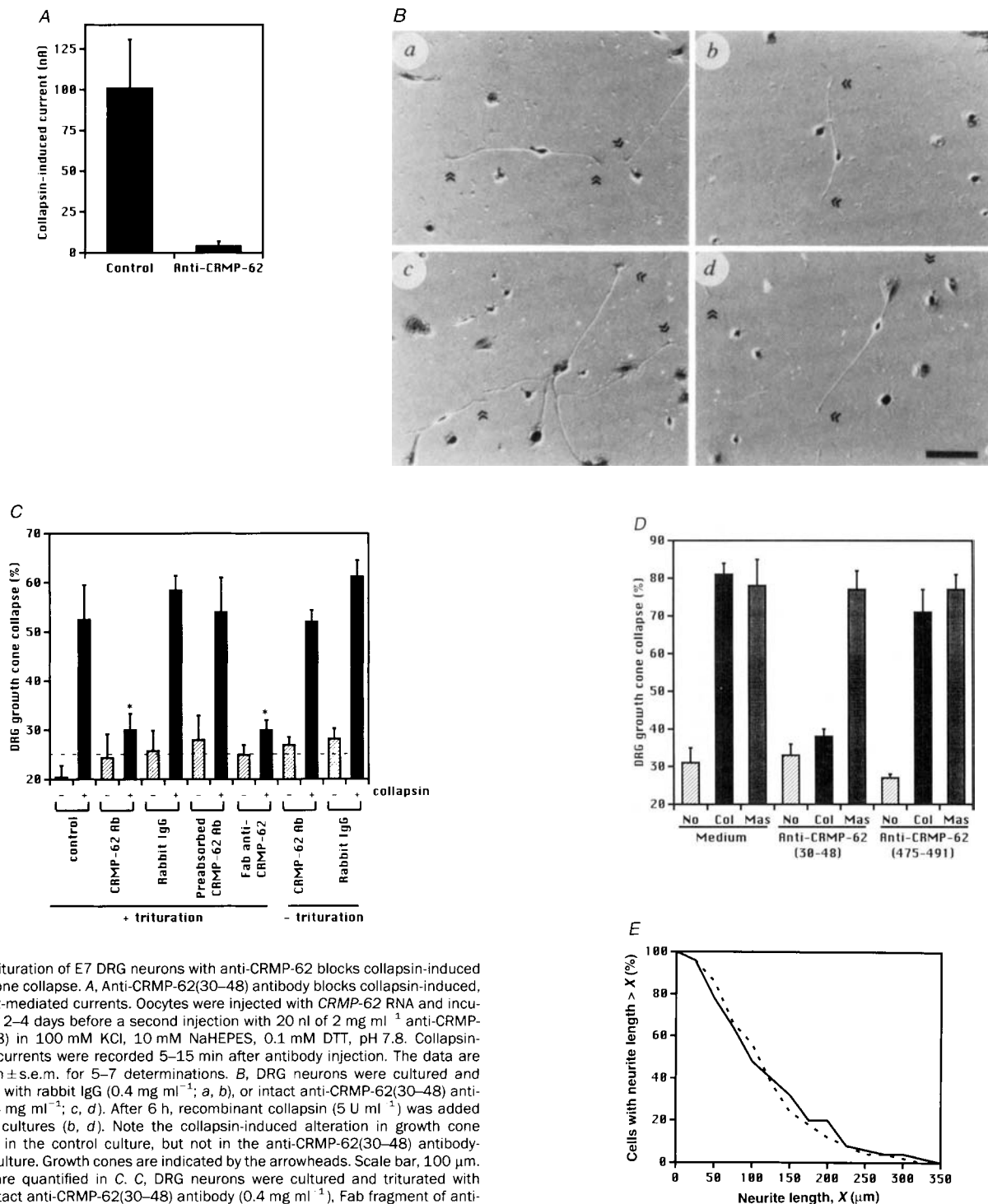


FIG. 4 Trituration of E7 DRG neurons with anti-CRMP-62 blocks collapsin-induced growth cone collapse. **A**, Anti-CRMP-62(30–48) antibody blocks collapsin-induced, CRMP-62-mediated currents. Oocytes were injected with *CRMP-62* RNA and incubated for 2–4 days before a second injection with 20 nl of 2 mg ml⁻¹ anti-CRMP-62(30–48) in 100 mM KCl, 10 mM NaHEPES, 0.1 mM DTT, pH 7.8. Collapsin-induced currents were recorded 5–15 min after antibody injection. The data are the mean $\pm$ s.e.m. for 5–7 determinations. **B**, DRG neurons were cultured and triturated with rabbit IgG (0.4 mg ml⁻¹; **a**, **b**), or intact anti-CRMP-62(30–48) antibody (0.4 mg ml⁻¹; **c**, **d**). After 6 h, recombinant collapsin (5 U ml⁻¹) was added to some cultures (**b**, **d**). Note the collapsin-induced alteration in growth cone structure in the control culture, but not in the anti-CRMP-62(30–48) antibody-treated culture. Growth cones are indicated by the arrowheads. Scale bar, 100 μ m. Results are quantified in **C**. **C**, DRG neurons were cultured and triturated with buffer, intact anti-CRMP-62(30–48) antibody (0.4 mg ml⁻¹), Fab fragment of anti-CRMP-62(30–48) antibody (0.1 mg ml⁻¹), rabbit IgG (0.4 mg ml⁻¹), or pre-absorbed anti-CRMP-62(30–48) antibody. The fraction of collapsed growth cones after 30 min exposure to buffer or 5 U ml⁻¹ collapsin is presented as the mean $\pm$ s.e.m. for 4–6 separate experiments. The anti-CRMP-62(30–48) antibody and its Fab fragment significantly reduced collapsin-induced growth cone collapse (asterisk, $P \leq 0.05$, Student's two-tailed *t* test), whereas control solutions did not. This antibody did not alter growth cone collapse when added without trituration. **D**, DRG neurons were cultured and triturated with buffer, anti-CRMP-62(30–48) antibody (0.2 mg ml⁻¹) or anti-CRMP-62(475–491) antibody (0.3 mg ml⁻¹). The fraction of collapsed growth cones after 30 min exposure to buffer (No), collapsin (Col, 5 U ml⁻¹) or mastoparan (Mas, 5 μ M) is presented as the mean $\pm$ s.e.m. for 6 separate experiments. The anti-CRMP-62(30–48) antibody significantly reduced collapsin-induced growth cone collapse ($P \leq 0.05$, Student's two-tailed *t* test), whereas the anti-CRMP-62(475–491) antibody did not. Neither antibody altered mastoparan-induced growth cone collapse. **E**, The extent of neurite outgrowth from DRG cells triturated with rabbit IgG (solid line) or the anti-CRMP-62(30–48) antibody (broken line) was determined after 5 h in culture in the absence of collapsin. The fraction of neurons with a total neurite length longer than *X* is plotted. No significant differences were detected in 4 separate experiments.

METHODS. Dissociated cell cultures were prepared from chick E7 DRG treated with 0.25% trypsin, 1 mM EDTA at 37 °C for 20 min⁸. The tissue was then triturated 30 times in growth medium using P200 plastic tips. The material was pre-plated on untreated plastic for 2 h to allow non-neuronal cells to attach, and then the floating cells were collected and triturated 70 times in F12 medium with 10 mM NaHEPES, pH 7.4 and various antibodies^{8,21}. After trituration, cells were diluted eightfold into growth medium and plated on laminin-coated glass slides. Fab fragments were created using immobilized papain (Pierce) and undigested antibody was removed by protein A-agarose absorption. Immunostaining for rabbit IgG was detectable in antibody-triturated, but not control, cells (not shown). Following 6 h at 37 °C, collapsin was added to the dissociated cells and the mixture was incubated at 37 °C for 30 min before fixation. The terminus of each neurite longer than 30 μ m was scored for growth cone collapse as in the explant cultures^{8,8}. The total outgrowth per neurite-bearing cell was quantified as described^{7,8,22}. The percentage of cells bearing neurites was 43 $\pm$ 6% in rabbit IgG-triturated cultures and 50 $\pm$ 6% in anti-CRMP-62(30–48) antibody-triturated cultures (mean $\pm$ s.e.m., *n* = 4).

the antibody blocks collapsin-induced currents (Fig. 4A). Trituration of DRG neurons with anti-CRMP-62(30–48) blocks subsequent collapsin-induced growth cone collapse (Fig. 4B, C). Rabbit IgG and preabsorbed anti-CRMP-62(30–48) antibody have no effect. Addition of anti-CRMP-62(30–48) after trituration does not prevent collapsin-induced collapse. The Fab fragment of anti-CRMP-62(30–48) is as effective as intact immunoglobulin (Fig. 4C), indicating that cross-linking and aggregation of antigen do not account for these effects. Trituration with anti-CRMP-62(30–48) has no effect on neuronal survival, neurite formation or extension in the absence of collapsin (Fig. 4E).

We also trituated cells with anti-CRMP-62(475–491). This portion of CRMP-62 is not conserved with UNC-33 or between hCRMP-1 and hCRMP-2, and is not included in the original truncated clone isolated by oocyte expression. The anti-CRMP-62(475–491) antibody does not block collapse induced by collapsin or mastoparan (Fig. 4D). This verifies that the 475–491 region is not critical for CRMP-62 function, and that blockade of collapsin action by anti-CRMP-62(30–48) cannot be explained as a nonspecific effect of antibody colocalization with CRMP-62. Mastoparan stimulates PTX-sensitive G proteins and induces growth cone collapse in control cultures^{6,8} and those trituated with anti-CRMP-62(30–48) antibody (Fig. 4D). Thus, those elements of the collapse pathway which are downstream of G protein activation are not disrupted by the anti-CRMP-62(30–48) antibody.

The hypothesis that collapsin activates a G protein signalling cascade is based on several findings: pertussis holotoxin blocks collapsin effects; collapsin activates an oocyte Ca^{2+} -sensitive chloride channel; and G protein activation collapses growth cones. CRMP-62 is likely to mediate or facilitate an interaction between a collapsin-binding transmembrane receptor and intracellular G proteins. In such a model, CRMP-62 may be an intracellular component of a multisubunit receptor, or may act as an intermediary between receptor and G protein. CRMP-62 cannot act downstream of the G protein because the response to ligands for other G protein-coupled receptors is not altered by CRMP-62, and because mastoparan-induced collapse is not blocked by anti-CRMP-62(30–48).

Generally, G protein-coupled receptors contain seven membrane-spanning domains and couple directly to G proteins. A collapsin-binding protein has not yet been identified, but its apparent requirement for CRMP-62 and its ability to respond to a macromolecular ligand which can exist in membrane-bound forms^{2–4} imply that it has unique characteristics. Whether CRMP-62 and/or a collapsin-binding protein interact directly or indirectly with a G protein remains to be determined. It is clear that CRMP-62 is expressed selectively in developing nervous system, is capable of reconstituting a collapsin-regulated G protein cascade in oocytes, and is essential for collapsin-induced DRG growth cone collapse. Further analysis of the protein-protein interactions of CRMP-62 should lead to a more complete understanding of the molecular mechanism whereby repulsive axon guidance clues are transduced into changes in growth cone motility. □

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Channel gating governed symmetrically by conserved leucine residues in the M2 domain of nicotinic receptors

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IN nicotinic acetylcholine receptors (nAChR), as well as glycine, GABA_A (γ -aminobutyric acid), serotonin (5-HT₃), and GluCl glutamate receptors, a leucine residue at the approximate midpoint of the M2 transmembrane domain (the 9' position¹) is conserved across most known subunits². Structural data for the nAChR suggest that the Leu 9' residues occupy a 'kink' in each of the five M2 helices and point into the closed channel; in the opening step, the M2 helices rotate so that Leu 9' side chains no longer occlude the conduction pathway³. Mutation of Leu 9' to one of several other residues slows desensitization and increases sensitivity to agonist^{4–6}. We have exploited the $\alpha_2\beta\gamma\delta$ stoichiometry of muscle nAChR to express receptors with $m_s^* = 0$ to 5 Leu 9'Ser mutated subunits. Strikingly, each Leu 9'Ser mutation shifts the dose-response relation for ACh to the left by ~10-fold; a nAChR with $m_s^* = 4$ is 10⁴-fold more sensitive than the wild type. The results suggest that each of the five Leu 9' residues participates independently and symmetrically in a key step in the structural transition between the closed and open states.

Oocytes were injected with mRNA for wild-type subunits or for subunits containing the Leu 9'Ser mutation; the latter are denoted here with an asterisk. Figure 1 shows raw traces and dose-response data for nAChRs that exemplify combinations for $m_s^* = 1, 2, 3$ and 4 Leu 9'Ser mutations, respectively. The clear trend is that receptors with increasing m_s^* require decreased ACh concentrations for half-maximum response (EC_{50}). We analysed these dose-response relations for all four possible hetero-oligomeric combinations with $m_s^* = 1$ and for all but one of the combinations yielding $m_s^* = 2, 3, 4$ and 5 that contain either 2 α or 2 α^* subunits (Table 1; the $\alpha_2\beta^*\gamma^*\delta$ combination gave no detectable expression). The EC_{50} for ACh decreases by ~10-fold for each additional mutated subunit (Fig. 1c). Thus the wild-type nAChR has an EC_{50} of 24 μM , and the two combinations tested with $m_s^* = 4$ have EC_{50} of 2.0 and 2.3 nM.

There is a range of up to fourfold among the EC_{50} values within some groups with equal m_s^* . These extrema were not consistently associated with a particular mutated subunit.

Dose-response relations for the $\alpha^*\alpha\beta\gamma\delta$ receptor (in the $m_s^* = 1$ group) were determined by injecting oocytes with mixtures of α and α^* messenger RNA, along with β , γ and δ (for example,

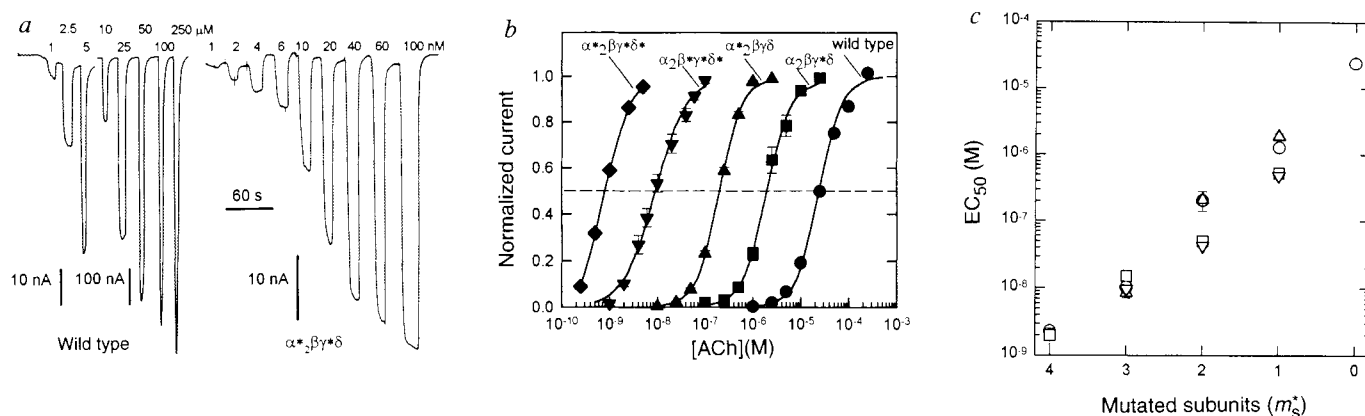


FIG. 1 *a*, Voltage-clamp currents from oocytes expressing mouse muscle acetylcholine receptors (AChR). Left, wild type; right, a subunit combination with 3 Leu 9/Ser mutations. ACh concentrations for each application are shown above the traces. *b*, Normalized average dose-response relations for exemplary combinations containing 0 (wild type) to 4 ($m_s^*=4$) Leu 9/Ser mutations: $\alpha_2\beta\gamma\delta$ (wild type), $\alpha\beta\gamma^*\delta$, $\alpha_2\beta^*\gamma\delta$, $\alpha_2\beta\gamma^*\delta$ and $\alpha^*\beta\gamma\delta$. The horizontal line at 0.5 represents the EC_{50} for each combination. The receptors with $m_s^*=4$ expressed rather low maximal current levels (20–100 nA). Each dose-response relation represents at least 5 oocytes from at least 2 batches. *c*, Relationship between number of mutated subunits and EC_{50} (logarithmic axis) for all the combinations measured (Table 1). Each symbol represents a distinct combination. S.e.m.s are shown where they exceed the size of the symbols.

METHODS. Leucine-to-serine mutations were generated by site-directed mutagenesis using the Clontech Transformer site-directed mutagenesis kit (Palo Alto, CA) and confirmed by sequencing. mRNA was synthesized

in vitro using the Megascript kit (Ambion, Austin, TX). pBluescript plasmids containing the AChR subunits were linearized and run-off transcripts prepared with T7 RNA polymerase Stage V-VI *Xenopus* oocytes were isolated and injected with 10–50 ng of mRNA in a stoichiometric ratio for $\alpha:\beta:\gamma:\delta$ of 2:1:1:1 (ref. 12). Before recording, oocytes were incubated at 18 °C in a modified Barth's solution supplemented with 50 $\mu\text{g ml}^{-1}$ Gentamicin, 2.5 mM pyruvate and 0.6 mM theophylline. Electrophysiological recordings were carried out 2–4 days after injection. Membrane potential was held at –80 mV with a 2-electrode voltage-clamp circuit. Bath solutions contained 96 mM NaCl, 2 mM KCl, 1 mM MgCl_2 and 5 mM HEPES, pH 7.5. Ca^{2+} was omitted from the bath solution and atropine (1 μM) was included to prevent activation of endogenous Ca^{2+} -activated Cl^- channels via muscarinic receptors. Individual dose-response relations were fitted to the Hill equation, $I/I_{\text{max}} = 1/(1 + \{\text{EC}_{50}/[\text{A}]\}^{n_H})$, where $[\text{A}]$ is the ACh concentration, EC_{50} is the ACh concentration giving half-maximal response, I_{max} is the maximal response, and n_H is the Hill coefficient.

Fig. 2). We analysed the responses assuming that (1) currents summed from independent populations of $\alpha_2\beta\gamma\delta$, $\alpha_2\beta^*\gamma\delta$ and $\alpha^*\alpha\beta\gamma\delta$ receptors, (2) receptors assembled equally well with α and with α^* , and (3) the two possible subunit arrangements for $\alpha^*\alpha\beta\gamma\delta$ receptors had identical responses. The calculated EC_{50} values for $\alpha^*\alpha\beta\gamma\delta$ ranged from 1 to 2 μM , within the range observed for the other receptors with $m_s^*=1$. These results, and the observation that the $\alpha_2\beta\gamma\delta$ receptor has an EC_{50} in the range of other receptors with $m_s^*=2$, show that the α Leu 9' residues do not occupy a privileged position in the gating process, despite the fact that agonist binds at least partly to the α -subunit.

TABLE 1 Dose-response relations for mouse muscle ACh receptors containing various numbers of mutated Leu 9/Ser subunits (m_s^*)

m_s^*	mRNA injections	EC_{50} (nM)	Hill coefficient
0	$\alpha_2\beta\gamma\delta$	24,010	1.68
1	$\alpha^*\alpha\beta\gamma\delta$	1,290	2.15 ± 0.22
	$\alpha_2\beta^*\gamma\delta$	531	2.03
	$\alpha_2\beta\gamma^*\delta$	1,910	1.82 ± 0.14
	$\alpha_2\beta\gamma\delta^*$	486	1.98
2	$\alpha_2^*\beta\gamma\delta$	202	1.81
	$\alpha_2\beta^*\gamma^*\delta$	49.7	1.64
	$\alpha_2\beta^*\gamma\delta^*$	208 ± 69	1.34
	$\alpha_2\beta\gamma^*\delta^*$	42.7	1.89
3	$\alpha_2^*\beta^*\gamma\delta$	10.3	1.44
	$\alpha_2^*\beta\gamma^*\delta$	15.1	1.61
	$\alpha_2^*\beta\gamma\delta^*$	8.4 ± 1.3	1.45
	$\alpha_2\beta^*\gamma^*\delta^*$	9.8 ± 1.3	1.30
4	$\alpha_2^*\beta^*\gamma^*\delta$	2.3	0.96
	$\alpha_2^*\beta\gamma^*\delta^*$	2.0 ± 0.6	1.02 ± 0.26
5	$\alpha_2^*\beta^*\gamma^*\delta^*$	<1	—

S.e.m.s for EC_{50} were less than 10% of the mean, except where given; s.e.m.s for Hill coefficient were less than 0.07, except where given. Responses for the $\alpha_2^*\beta\gamma^*\delta^*$ combination were too small for reliable measurements of EC_{50} or Hill coefficient.

In single-channel studies with the $\alpha_2^*\beta\gamma\delta$ receptor (Fig. 3), ACh evoked bursts of openings lasting hundreds of milliseconds, similar to records with the α Leu 9 Cys AChR (ref. 7) and much longer than bursts in the wild type at the same ACh concentration (ACh). Within a burst, however, there were many brief (time constant ~ 0.15 ms) closings, so that the longest compo-

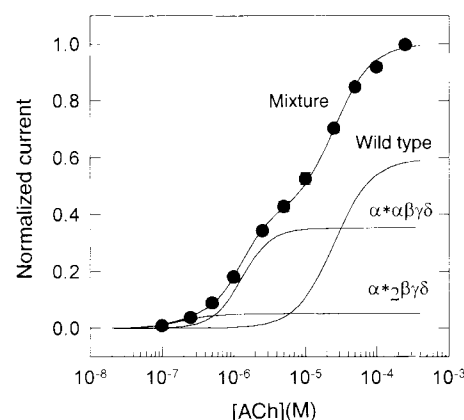


FIG. 2 Dose-response relation for $\alpha^*\alpha\beta\gamma\delta$, determined by injecting oocytes with a mixture of mRNA for α^* , α , β , γ and δ subunits. The injected mRNA mole fraction $\alpha^*/(\alpha^* + \alpha)$ was 0.5. The dose-response relations were measured independently for $\alpha_2^*\beta\gamma\delta$ and for $\alpha_2\beta\gamma\delta$ in other oocytes from the same batch. The normalized currents for the mixture were expressed as the sum of dose-response relations for these 2 combinations plus a third relation for $\alpha\alpha^*\beta\gamma\delta$. With the assumptions given in the text, if the mole fraction of expressed α^* subunit is a^* , the proportion of receptors of each species is $(a^*)^2$, $(1-a^*)^2$, and $2a^*(1-a^*)$, respectively. The results fit best for $a^*=0.25$. The calculated dose-response relation for $\alpha^*\alpha\beta\gamma\delta$ is characterized by $\text{EC}_{50}=1.29 \mu\text{M}$ and $n_H=2.15$.

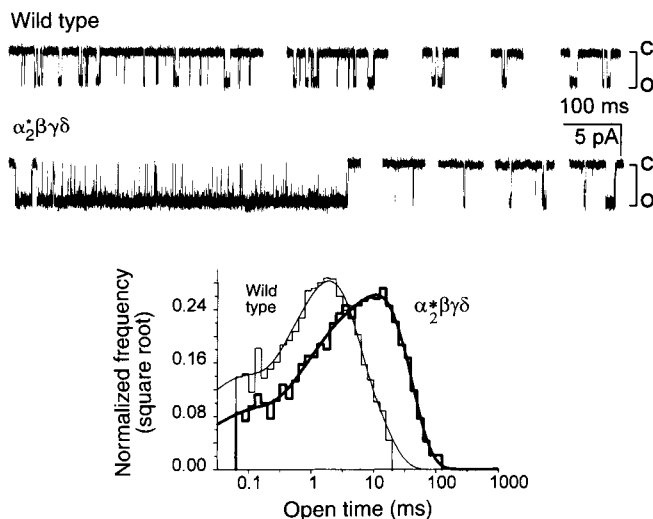


FIG. 3 Single-channel records from wild-type and $\alpha_2\beta\gamma\delta$ receptors. Outside-out patches were exposed to 25 μM and 200 nM ACh, respectively ($\sim\text{EC}_{50}$ for both receptors) at -100 mV. The displayed traces were filtered at 5 kHz; gaps denote long closed times. The bottom panel shows open-time distributions. Smooth curves are fits to the time constants and normalized frequencies, as follows. Wild-type: 0.05 ms, 12%; 1.7 ms, 70%; 4.4 ms, 18%; $\alpha_2\beta\gamma\delta$: 0.08 ms, 5%; 1.9 ms, 18%; 11.4 ms, 77%. Bins have logarithmically increasing durations, and frequencies are plotted as square roots¹³. These procedures resolved event durations >60 μs .

METHODS. mRNA was generated from complementary DNA clones in pAMV (ref. 14). Bath and pipette solutions contained 100 mM KCl, 2 mM MgCl_2 , 2 mM BaCl_2 , 10 mM HEPES, 10 mM EGTA, 1 μM atropine, pH 7.4. Recordings were made with a GeneClamp 500/CV-5 instrument and analysed with pCLAMP software (Axon Instruments, Foster City, CA).

ment of open time was less than threefold longer for the $\alpha_2\beta\gamma\delta$ receptor than for the wild-type receptor. In a simple structural explanation of these data, mutant channels with two bound ACh molecules would remain open roughly as long as normal channels, then close, then rapidly reopen because the axial cluster of side chains that closes the channel³ is less stable with Ser than with Leu. Other interpretations are possible⁵. Single-channel current amplitudes (~ 6 pA at -100 mV in symmetrical KCl solutions) differed by $<10\%$ among the receptors tested ($\alpha_2\beta\gamma\delta$, $\alpha_2\beta\gamma^*\delta$, $\alpha_2^*\beta\gamma\delta$ and $\alpha_2\beta^*\gamma\delta$).

Average Hill coefficients for the groups with $m_s^* = 1, 2$ and 3 were 2.00 ± 0.07 , 1.67 ± 0.12 and 1.45 ± 0.06 respectively (mean $\pm$ s.e.m.; $n > 20$; the Hill coefficients obtained for $m_s^* = 0$ and for $m_s^* = 4$ were not compared in this series because they could be unreliable owing to desensitization and to very small responses, respectively). This trend suggests that, for the mutated channels as for the wild type, the open state is more likely to be associated with the presence of two bound ACh molecules, but perhaps with an increasing contribution from unliganded or singly liganded receptors as m_s^* increases. Consistent with this idea, single-channel recordings showed a higher frequency of openings in the absence of ACh⁸ for the $\alpha_2\beta\gamma\delta$ and $\alpha_2^*\beta\gamma\delta$ receptors than for the wild-type receptor (results not shown).

Receptors with $m_s^* = 0$ and 1 desensitized in the presence of ACh concentrations $\geq 0.2 \times \text{EC}_{50}$ and $\geq 1 \times \text{EC}_{50}$, respectively. Combinations with $m_s^* \geq 2$ showed no macroscopic desensitization for [ACh] at concentrations up to $5 \times \text{EC}_{50}$, with the exception of $\alpha_2^*\beta\gamma\delta$, which desensitized at $[\text{ACh}] \geq 3 \times \text{EC}_{50}$. These data agree with previous observations that Leu 9' mutations of homooligomeric ACh and 5-HT₃ receptors reduce the ratio of desensitized to activated receptors^{4,6}.

That Leu 9'Ser mutations are nearly independent, equivalent, and multiplicative in their effect on the dose-response relations reinforces the general view that the five M2 domains move independently and symmetrically during AChR gating³,

although the energy for channel gating derives from contacts at only two binding sites. More specifically, our data support the suggestion that contacts involving each of the five Leu residues under consideration—one in each M2 region—play a key role in gating³. The 10-fold difference in EC_{50} for each mutation suggests that each contact contributes a free energy difference of ~ 1.4 kcal mol^{-1} to the ensemble of steps between binding of the receptor and opening of the channel.

Mutations at many M2 positions affect gating^{4,5,9,10,15}. The suggestion that Leu 9' plays a unique role in receptor function³ led us to examine the consequences of mutating Leu 16', which is also well conserved among AChR subunits and would lie on the same face of an α -helix as the 9' position. ($\alpha\text{Leu } 16'\text{Ser}$), $\beta\gamma\delta$ receptors had EC_{50} values of 320 nM, near that for the corresponding Leu 9'Ser mutation. However, Leu 16'Ser mutations in β - and γ -subunits had little effect by themselves on dose-response relations, and none of the non- $\alpha\text{Leu } 16'\text{Ser}$ subunits produced further shifts in the dose-response relation when combined with the $\alpha\text{Leu } 16'\text{Ser}$ subunit. Thus the symmetrical interactions involving the M2 domains do not extend to the 16' position.

These data imply that the AChR and homologous receptors for other transmitters have not evolved to maximize either the affinity for transmitter or the open-state lifetime. Responses to low transmitter concentrations are inappropriate, given the high concentration of transmitter in the synaptic cleft at the start of transmission¹¹; and long bursts of openings would vitiate rapid signal processing. Chemical synapses involving ligand-gated channels apparently evolved as exquisite electrochemical machines, specialized to function on a timescale of milliseconds and a distance scale of micrometres; and this optimization is evident even at the level of individual side chains on receptor proteins.

Note added in proof: Similar data have been obtained for Leu9'Thr mutations (G. Filatov and M. M. White *Molec. Pharmacol.*, in the press). □

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Angiogenesis mediated by soluble forms of E-selectin and vascular cell adhesion molecule-1

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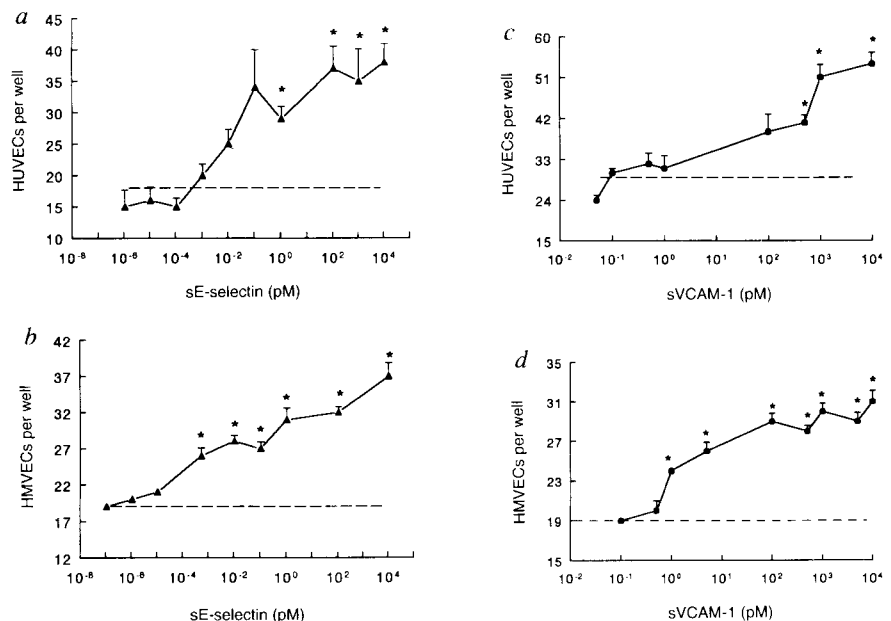
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ENDOTHELIAL adhesion molecules facilitate the entry of leukocytes into inflamed tissues. This in turn promotes neovascularization, a process central to the progression of rheumatoid arthritis, tumour growth and wound repair¹. Here we test the hypothesis that soluble endothelial adhesion molecules promote angiogenesis²⁻⁴. Human recombinant soluble E-selectin and soluble vascular cell adhesion

molecule-1 induced chemotaxis of human endothelial cells *in vitro* and were angiogenic in rat cornea. Soluble E-selectin acted on endothelial cells in part through a sialyl Lewis-X-dependent mechanism, while soluble vascular cell adhesion molecule-1 acted on endothelial cells in part through a very late antigen (VLA)-4 dependent mechanism. The chemotactic activity of rheumatoid synovial fluid for endothelial cells, and also its angiogenic activity, were blocked by antibodies to either soluble E-selectin or soluble vascular cell adhesion molecule-1. These results suggest a novel function for soluble endothelial adhesion molecules as mediators of angiogenesis.

First, we showed that recombinant human soluble E-selectin stimulated chemotaxis of human umbilical vein endothelial cells (HUVECs) (Fig. 1a) and human dermal microvascular endothelial cells (HMVECs) (Fig. 1b). Soluble E-selectin was potentially chemotactic for HUVECs in the picomolar range, with 0.01 pM soluble E-selectin inducing chemotaxis equivalent to 60 nM control angiogenic cytokine basic fibroblast growth factor (bFGF). Recombinant human soluble vascular-cell-adhesion molecule-1 (soluble VCAM-1) was also chemotactic, with 1 nM soluble VCAM-1 inducing HUVEC chemotaxis equivalent to 60 nM bFGF (Fig. 1c). Similar results were obtained with HMVECs (Fig. 1d), indicating that these effects were not restricted to HUVECs. Checkerboard analysis, incorporating varying concentrations of chemoattractant in the upper and lower chemotaxis chambers, indicated that the effects of soluble

FIG. 1 Chemotaxis of endothelial cells. Chemotaxis of HUVECs or HMVECs (Clonetics, San Diego) was performed in 48-well blind-well chemotaxis chambers with polycarbonate membranes of 8 μ m pore size (Neuroprobe, Cabinjohn, MD)^{5,17}. HUVECs (2.5×10^4 cells per well) or HMVECs (3.75×10^4 cells per well) in 25 μ l of Roswell Park Memorial Institute (RPMI) media containing +0.1% fetal calf serum were added to the bottom wells of the chambers. Inverted chambers were incubated at 37 °C for 2 h, allowing endothelial cell attachment. The following were added to the top half of reinverted chambers: PBS with or without recombinant human soluble E-selectin (sE-selectin) (a, b), or soluble VCAM-1 (sVCAM-1) (c, d) (Biogen, Cambridge, MA), or bFGF (60 nM) (R&D Systems, Minneapolis). The sE-selectin was biologically active, functioning as an adhesion molecule². This binding was blocked by EDTA (R. Lobb, personal communication). After incubation for 2 h, the membranes were removed, fixed in methanol and stained with Diff-Quik (Baxter Diagnostics, Chicago). Each test group was assayed in quadruplicate. Three high-power microscope fields were counted in each replicate well and results were expressed as cells per well. Statistical analysis was done with an unpaired Student's *t*-test without correcting for multiple comparisons. Values significantly different ($P < 0.05$) from the PBS control (horizontal broken line) are indicated by stars. Chemotaxis in response to bFGF was: a, 25 ± 2 cells per well ($\pm$ s.e.m.); b, 37 ± 2 ; c, 48 ± 1 ; d, 31 ± 1 . Endotoxin concentrations in the soluble adhesion preparations did not exceed 0.04 EU mg^{-1} in the limulus amoebocyte lysate assay (Associates of Cape Cod, Woods Hole, MA). PBS controls containing up to 0.4 EU mg^{-1} endotoxin did not affect the assay. Results represent 1 of at least 3 experiments. e, f, The results of representative checkerboard analyses using HUVECs and varying concentrations of sVCAM-1 or sE-selectin in the upper or lower compartment of the chemotaxis chamber²⁰. Both sE-selectin (e) and sVCAM-1 (f) induced chemotactic rather than chemokinetic responses. Results are expressed as the number of cells $\pm$ s.e.m. per replicate well.



e

Lower	Upper			
	0	0.5 pM	110 pM	1.1 nM
0	19.3 $\pm$ 0.2	23.0 $\pm$ 1.2	28.5 $\pm$ 1.1	27.3 $\pm$ 2.1
0.5 pM	19.3 $\pm$ 0.7	20.8 $\pm$ 1.2	22.5 $\pm$ 1.1	27.3 $\pm$ 1.4
110 pM	17.3 $\pm$ 0.2	22.0 $\pm$ 1.7	26.3 $\pm$ 0.8	25.0 $\pm$ 1.6
1.1 nM	16.5 $\pm$ 1.2	22.3 $\pm$ 1.5	24.3 $\pm$ 1.0	22.8 $\pm$ 0.4

f

Lower	Upper			
	0	100 pM	1 nM	5 nM
0	13.3 $\pm$ 0.7	16.0 $\pm$ 1.1	25.8 $\pm$ 1.6	29.0 $\pm$ 2.8
100 pM	13.0 $\pm$ 0.7	11.8 $\pm$ 0.5	21.0 $\pm$ 1.1	28.5 $\pm$ 1.7
1 nM	12.8 $\pm$ 1.2	14.0 $\pm$ 0.9	13.3 $\pm$ 1.1	27.5 $\pm$ 2.2
5 nM	11.7 $\pm$ 0.5	14.3 $\pm$ 0.7	12.5 $\pm$ 0.4	12.3 $\pm$ 0.2

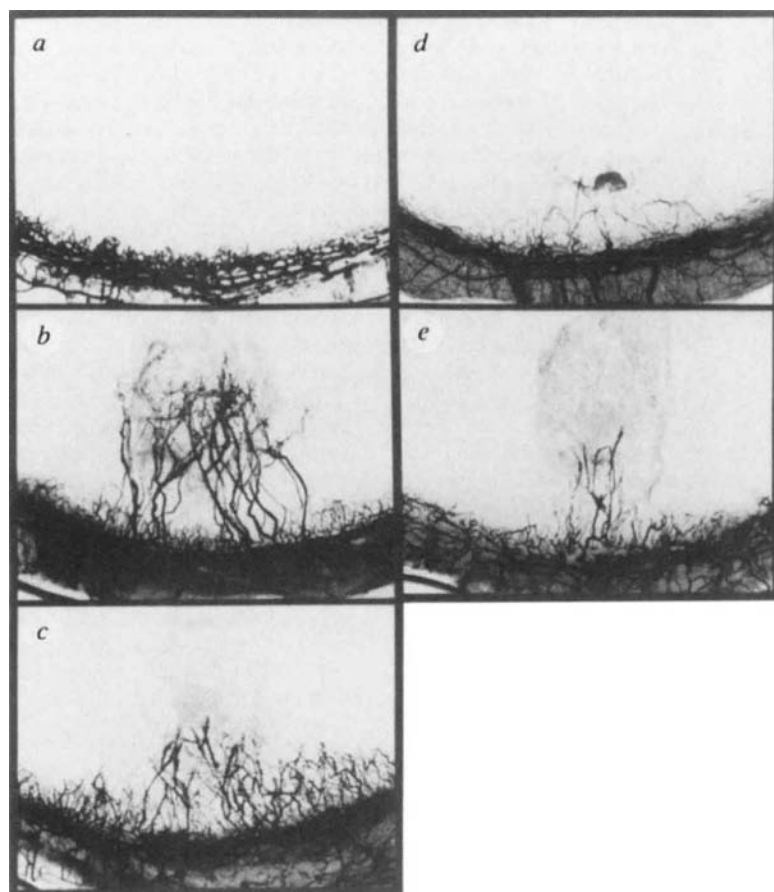


FIG. 2 a, Negative angiogenic response induced control PBS; b, positive angiogenic response induced by soluble E-selectin (10 nM); c, positive angiogenic response induced by soluble VCAM-1 (10 nM); d, minimal angiogenic response induced by rheumatoid arthritis (RA) synovial fluids (SFs) incubated with anti-E-selectin; e, markedly suppressed angiogenic response induced by RA SFs incubated with anti-VCAM-1.

METHODS. Test substances were combined 1:1 with Hydron (Interferon Sciences, New Brunswick, NJ) and implanted into the normally avascular corneal stroma of the rat several mm from the limbus^{5,21}. Corneas were perfused with colloidal carbon after 7 days to provide a permanent record of the angiogenic response (33 $\times$). No cornea exhibited histological evidence of nonspecific inflammation. All human samples were obtained with Institutional Review Board approval. SFs were obtained from arthrocentesis of patients with RA. SFs were diluted 1:50 with PBS and incubated with either anti-E-selectin, anti-VCAM-1, or isotype-matched control monoclonal antibody (mAb) for 1 h at 37 $^{\circ}$ C. The mAbs used were: mAb BB11, which recognizes E-selectin; mAb 4B9, which recognizes VCAM-1 domain 1; and mAb GH12, which recognizes VCAM-1 domain 4 (refs 22, 23). All test mAbs were used at 10 μ M ml⁻¹ and obtained from Biogen (Cambridge, MA). Control mAbs (10 μ M ml⁻¹) were obtained from Coulter Diagnostics (Hialeah, FL); mAbs 4B9 and GH12 were used simultaneously.

E-selectin or soluble VCAM-1 were chemotactic, not chemokinetic, for HUVECs or HMVECs (Fig. 1e, f). Neither soluble adhesion molecule (SAM) induced mitogenesis of HUVECs or HMVECs, suggesting that they mediated angiogenesis independently of stimulating endothelial cell proliferation (results not shown).

We next determined whether soluble E-selectin and soluble VCAM-1 were angiogenic *in vivo*. SAMs were incorporated into Hydron pellets and implanted into rat corneas. Soluble E-selectin (10 nM) or soluble VCAM-1 (10 nM) induced an angiogenic response in 3 out of 4 and 4 out of 4 corneas, respectively (Fig. 2a–c).

To determine whether the SAMs were acting *in vivo* by inducing the release of angiogenic cytokines by HUVECs⁵, these cells were incubated with either soluble E-selectin (up to 1.1 nM) or soluble VCAM-1 (up to 50 nM), at concentrations that met or exceeded those necessary to induce HUVEC chemotaxis *in vitro*.

Treatment of HUVECs with these SAMs did not increase their production of interleukin-8 (IL-8), tumour necrosis factor- α (TNF- α), or bFGF, as measured by enzyme-linked immunosorbent assay (ELISA), indicating that they did not act as indirect angiogenic mediators (results not shown).

To investigate the mechanism of action of soluble E-selectin on endothelial cells, HUVECs were incubated with a monoclonal antibody to the E-selectin ligand, sialyl Lewis-X, which is expressed on a variety of cells, including endothelial cells^{6–8} (Fig. 3). We detected the sialyl Lewis-X antigen on HUVECs by immunoperoxidase histochemistry (results not shown). In the presence of anti-sialyl Lewis-X, chemotaxis of HUVECs in response to soluble E-selectin was significantly reduced ($P < 0.05$), indicating that soluble E-selectin induces HUVEC chemotaxis in part through binding endothelial sialyl Lewis-X. Similar experiments involved incubating HUVECs with monoclonal antibodies to the soluble VCAM-1 ligand VLA-4, which

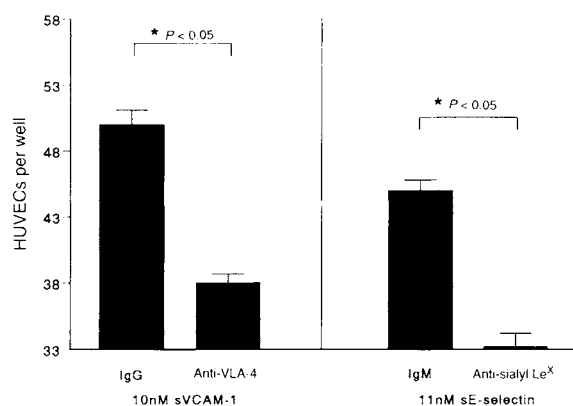
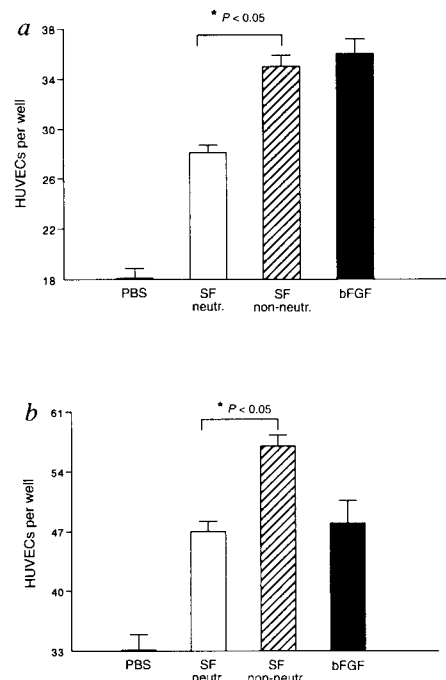


FIG. 3 Soluble E-selectin and soluble VCAM-1 mediate HUVEC chemotaxis by sialyl Lewis-X and VLA-4-dependent mechanisms, respectively. **METHODS.** We determined whether HUVECs migrated in response to sE-selectin or sVCAM-1 when the ligands for these molecules on target HUVECs were blocked. RPMI (0.5 ml) containing 10% fetal calf serum and 10 μ M ml⁻¹ mAb or isotype-matched control mAb were placed in replicate wells of confluent HUVEC-containing 24-well plates. The mAbs used were: mAb CSLEX1, which recognizes sialyl Lewis-X (Le^x) (Becton Dickinson, San Jose, CA); and mAb HP 1/2, which recognizes the α chain of VLA-4 (Biogen)²⁴. Isotype-matched control mAbs were obtained from Coulter Diagnostics. After incubation for 1 h at 37 $^{\circ}$ C in a humidified incubator gassed with 5% CO₂, HUVECs were collected and resuspended in RPMI containing 0.1% fetal calf serum and 10 μ M ml⁻¹ mAb, and chemotaxis was performed as described in Fig. 1. Results represent 1 of 3 experiments, each performed in quadruplicate. Additionally, in 3 experiments, concentrations of sE-selectin down to 1.1 nM or sVCAM-1 down to 5 pM showed similar results. Statistical analysis was done with an analysis of variance²⁵.

FIG. 4 Soluble E-selectin and soluble VCAM-1 account for a large portion of the RA SF chemotactic activity for HUVECs.

METHODS. RA SFs were obtained from 9 patients, diluted 1:50 with PBS and depleted of sE-selectin or sVCAM-1 as described in Fig. 2. Chemotaxis with SFs was done as described in Fig. 1. SF neutr., neutralized RA SFs; SF non-neutr., SFs incubated with isotype-matched control mAbs. Results represent the means $\pm$ s.e.m. for 9 patients. Depleted SFs contained no detectable antigenic sE-selectin or sVCAM-1 as measured by ELISA (R&D Systems, Minneapolis) (results not shown). Anti-E-selectin (a) or anti-VCAM-1 (b) completely eliminated the chemotactic activity for HUVECs induced by 11 nM sE-selectin or 10 nM sVCAM-1, respectively. None of the mAbs had any effect on bFGF-induced HUVEC chemotaxis.



is also expressed on endothelial cells and was detected on HUVECs by immunoperoxidase histochemistry (results not shown)⁹. Incubation of HUVECs with anti-VLA-4 significantly diminished ($P < 0.05$) the chemotactic activity for HUVECs in response to soluble VCAM-1, indicating that soluble VCAM-1 was acting on HUVECs to induce their chemotaxis in part by a VLA-4-dependent mechanism (Fig. 3).

We then determined whether SAMs contributed to the angiogenic activity of rheumatoid synovial fluid. Incubation with anti-E-selectin or anti-VCAM-1 significantly attenuated the rheumatoid synovial fluid chemotactic activity for HUVECs (Fig. 4a, b). Rheumatoid synovial fluid (5 μ g protein) was potently angiogenic in all 3 corneas examined. Neutralization of soluble E-selectin or soluble VCAM-1 diminished or completely abolished the angiogenic response (1 out of 11 positive corneas (Fig. 2d), and 2 out of 14 positive corneas and 3 out of 14 more or less weakly positive corneas (Fig. 2e), respectively). Incubation with control isotype-matched monoclonal antibody had no effect on the angiogenic activity present in the synovial fluid (6 out of 6 positive corneas). These results indicate that soluble E-selectin and soluble VCAM-1 account for a large portion of the angiogenic activity found in rheumatoid synovial fluids.

Cellular adhesion and angiogenesis, often regarded as separate processes, may be linked. We have shown that the $\beta 3$ integrin subunit is expressed in rheumatoid arthritis angiogenesis-rich synovial tissue, but virtually absent in normal angiogenesis-deficient synovial tissue¹⁰. Interestingly, the adhesion molecule $\alpha \nu \beta 3$ integrin has recently been identified as a marker of angiogenic vascular tissue in wound granulation tissue¹¹. Paradoxically, addition of anti- $\alpha \nu \beta 3$ promoted tube formation in fibrin gels and inhibited endothelial cell proliferation on a fibrinogen matrix¹². Anti- $\alpha 2 \beta 1$ integrin enhanced the number of capillary tubes formed by HUVECs *in vitro*¹². Similarly, sialyl Lewis-X/A has been implicated in bovine capillary morphogenesis *in vitro*¹³.

It has been shown that E-selectin and VCAM-1 are upregulated both in rheumatoid synovial fluid *in situ* and in soluble form in synovial fluid from rheumatoid arthritis compared with osteoarthritis^{4,14-16}. Despite the increased quantities of SAMs in angiogenic disease states, their main known functions have included mediating cellular adhesion when immobilized to plastic, and, for soluble E-selectin, recruiting neutrophils *in vitro*^{2,3}.

We have shown that soluble E-selectin and soluble VCAM-1 are angiogenic in the 10 nM range in the cornea. This compares with amounts reported for the induction of angiogenesis by IL-8, TNF- α , aFGF and bFGF, angiotropin, angiogenin and vascular endothelial growth factor^{5,17-19}. The studies described here suggest a proangiogenic role for soluble E-selectin and soluble VCAM-1. It is possible that when leukocytes bind to endothelial cells with concomitant release of cytokines, these molecules expressed on endothelial cells are then shed. The shed molecules in turn bind adjacent endothelial cells via their respective ligands, sialyl Lewis-X and VLA-4, exert a direct angiogenic effect on these endothelial cells and mediate inflammation. Our results therefore demonstrate a link between cellular adhesion and angiogenesis, and suggest a novel function for soluble E-selectin and soluble VCAM-1 in angiogenesis. □

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Direct and continuous assessment by cells of their position in a morphogen gradient¹⁻⁵

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ACCORDING to the morphogen gradient concept¹⁻⁵, cells in one part of an embryo secrete diffusible molecules (morphogens) that spread to other nearby cells and activate genes at different threshold concentrations. Strong support for the operation of a morphogen gradient mechanism in vertebrate development has come from the biochemical experiments of Green and Smith^{6,7}, who induced different kinds of gene expression in amphibian blastula cells exposed to small changes in activin concentration. But the interpretation of these experiments has been complicated by recent reports⁸⁻¹⁰ that cells tested for gene expression 3 hours after exposure to activin fail to show the graded response previously reported at 15 hours^{6,7}, a result suggesting that cells recognize their position in a gradient by an indirect mechanism. Here we conclude from the *in situ* analysis of blastula tissue containing activin-loaded beads¹¹ that cells respond directly to changing morphogen concentrations, in a way that resembles a ratchet-like process.

To analyse early responses to an activin gradient, we had first to determine how soon gene activation can be seen following exposure to the morphogen. Figure 1 shows that the time of *Xbra*¹² RNA synthesis is related to the developmental age of responding cells, and not to the time of first exposure to activin, a conclusion reached previously for certain other kinds of gene expression¹³ or cell behaviour¹⁴ in amphibian embryos. Figure 1 also shows that, in animal caps first exposed to activin at stage 9.5 (ref. 15), *Xbra* expression can be detected by RNase protection within 1 hour. However, by *in situ* hybridization, a longer time is needed for *Xbra* expression to be seen at a distance from the activin source. When beads containing a high concentration of activin (strong beads; see legend to Fig. 1) are enclosed in stage-9 animal caps and sectioned for *in situ* hybridization as described before¹¹, *Xbra* RNA can be seen well separated from the beads by *Xbra*-negative cells 2 hours after bead implantation (Fig. 2c). Therefore within 2 hours a gradient of activin seems to have been established, and cells have been able to respond to their appropriate activin concentration.

We can now ask how cells respond to the forming morphogen gradient during the first 2 hours after activin exposure, while the gradient is still being established. This provides a test of whether cells respond to a morphogen gradient directly or only after complex interactions among themselves. We might expect there to be an early time when cells close to a strong signalling source

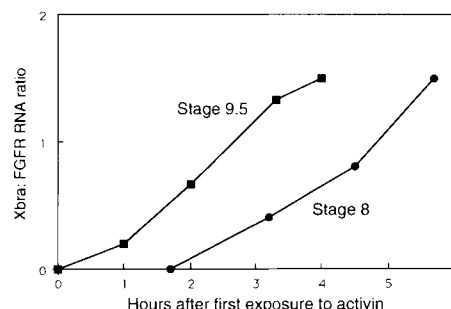


FIG. 1 The time course of *Xbra* expression is related to developmental stage and not to the time elapsed since activin exposure. Animal caps from stage 8 or stage 9.5 blastulae (separated by 1.5 h; ref. 15) were used to enclose activin-containing beads in sandwiches¹¹. These were cultured for the times shown when they were frozen for RNase protection analysis. Each sample was probed for *Xbra* and FGF receptor RNA, and analysed on the same gel. FGF receptor was used solely for quantitative comparison of samples. Affigel blue beads (Biorad) were incubated in a solution of 0.1% BSA containing human recombinant activin (17 nM, strong beads) for 30 min at 37 °C. They were then kept for up to 2 weeks at +4 °C until used. Each sandwich contained 5 beads.

would experience a low concentration of activin, such as would induce *Xbra*. After the time needed for activin concentration to build up, these same cells should have received too high a concentration of the morphogen to express *Xbra*. They should therefore discontinue *Xbra* expression and initiate a higher-grade response, such as *Xgsc*¹⁶, if they respond to the gradient in a simple and direct way. The time between activin bead implantation and the accumulation of enough *Xbra* messenger RNA to be seen by *in situ* methods is 1–2 hours. We have therefore tested the above proposition by removing beads at early times after implantation, and culturing disbeaded sandwiches until the normal time of *Xbra* expression about 2 hours later. We find that a 25-minute exposure to strong activin beads induces *Xbra* expression in cells close to where the beads were located (Fig. 2a). After a 50-minute exposure, the strongest *Xbra* expression has been extended away from the bead location (Fig. 2b), and an exposure of 2 hours or more results, as just described, in a ring of *Xbra*-positive cells separated from the bead location by several diameters of *Xbra*-negative cells (Fig. 2c–e). Activin-induced *Xgsc* expression is first seen 1–2 hours later than that of *Xbra*, and only in cells that are close to the bead location and that expressed *Xbra* at an earlier time (Fig. 2f–j). As previously observed¹¹, distal *Xgsc* expression overlaps with proximal *Xbra* expression (see also Fig. 3b, c). These are the results that we would expect of a simple and direct response of cells to a forming morphogen gradient.

We are now in a position to approach the problem of how cells recognize their position in a morphogen gradient, and specifically to ask whether they do so continuously or only at a particular time. To distinguish these ideas, we carried out a bead

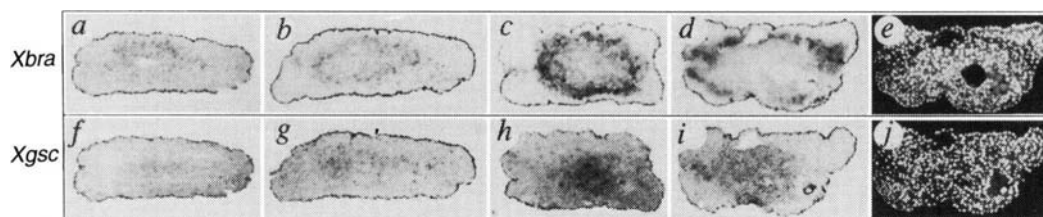


FIG. 2 Early spatial changes of expression of *Xbra* and *Xgsc* in activin-bead sandwiches. Strong beads (incubated in 15 nM activin) were implanted into stage 8.5 animal caps. Beads were removed after 25 min (a, f), 50 min (b, g) or 2 h (c, h). Disbeaded sandwiches were resealed for further culture. d, e, i, j, Samples fixed at 3 h. The total culture time

for all samples was 3–4 h, so that they had reached control stage 10.5 when fixed. Histological sections were processed by *in situ* hybridization, as described¹¹, for expression of *Xbra* (a–e) or *Xgsc* (f–j). e, j, Same sections as in d and i, but photographed to show nuclear staining, and therefore an even distribution of cells throughout the section.

FIG. 3 Cells can switch their gene response to a changing morphogen gradient. Weak beads, incubated in 1.5 nM activin, were implanted into animal cap sandwiches at stage 9. Two hours later (at control stage 10), some were fixed and *in situ* hybridized for *Xbra* expression (a). At the same time, others in the same series had their weak beads removed and replaced by strong beads. After a further 2 h (to control stage 10.75), they were fixed and *in situ* hybridized for expression of *Xbra* (b) or *Xgsc* (c), in nearby sections from the same conjugate.

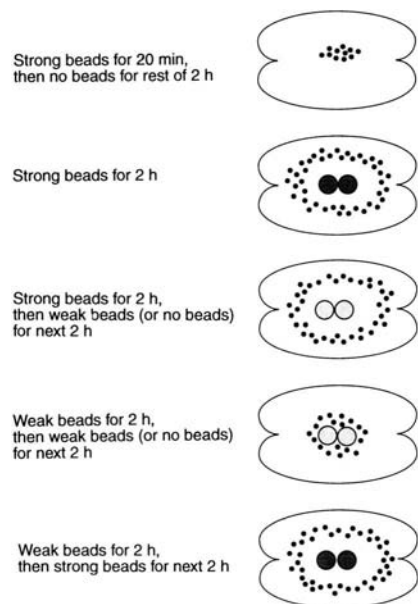
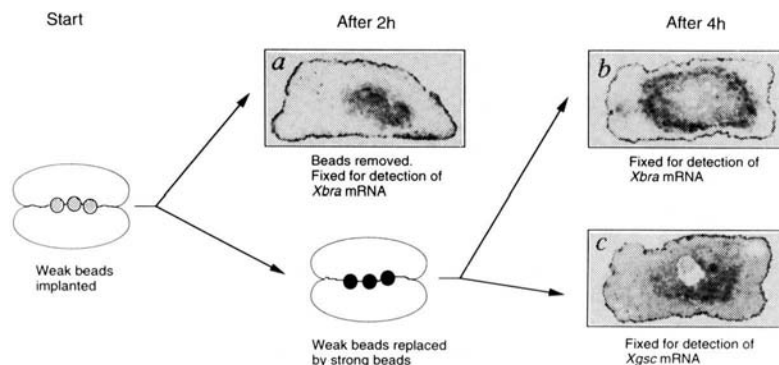


FIG. 4 Diagram to show the results of activin bead implant and exchange experiments. In each figure the outer line represents an animal cap sandwich. The small spots indicate *Xbra*-positive cells. The lower four figures show 2 activin beads in each sandwich. Strong (15 nM) beads are indicated by dark filled circles, and weak (1.5 nM) beads by light filled circles. Within the timescale of these experiments, cells can increase but not decrease their level of response, suggesting a ratchet mechanism. Our conclusions are not affected by whether the morphogen gradient is created by diffusion or by a relay process.

transfer experiment in two parts. First, weak beads were implanted into stage-9 sandwiches that were cultured for 2 hours when (at the equivalent of stage 10) they were fixed; *Xbra* expression was seen, as expected, in only those cells located close to the beads (Fig. 3a). The second part of the experiment was initiated as in the first part, but now the weak beads were replaced by strong beads after 2 hours, and the resulting sandwiches cultured for a further 2 hours (until stage 10.75). *In situ* analysis showed *Xbra* expression in a ring distant from the beads (Fig. 3b); notably, the cells nearest the beads were now *Xbra*-negative; they were also *Xgsc*-positive (Fig. 3c), as expected for response to a high activin concentration. It has previously been established that cells do not move significantly under these conditions¹¹. Therefore cells that have already made an early response to their position in a morphogen gradient can subsequently change their response to a higher level. We conclude that cells can assess morphogen concentration continuously, and not only at one particular time.

Lastly we have asked whether cells change their response in a downward as well as upward direction, and have carried out the series of bead replacement experiments summarized in Fig. 4. Conjugates exposed to weak or strong beads show the same response at 4 hours, whether or not beads are removed after 2 hours. But cells exposed first to weak beads and then to strong beads change their *Xbra* expression to the distal (strong) type, as described. In contrast, strong beads replaced by weak beads result in a continued strong-bead response. From the combination of our results (Fig. 4), we conclude that cells express genes characteristic of the highest concentration of morphogen to which they have been exposed within their period of

competence¹⁷, as if by a ratchet-like process. Whether activin or some other related molecule such as Veg1 (refs 18–20) is a natural mesoderm inducer, our results, which differ in design from those previously reported^{8, 10, 21, 22}, strongly suggest that cells interpret their position in a morphogen gradient in a remarkably simple and direct way. □

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Autocrine mitogenic activity of pheromones produced by the protozoan ciliate *Euplotes raikovi*

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DIFFUSIBLE polypeptide pheromones (formerly referred to as mating-type factors, sex factors or gamones), which distinguish otherwise morphologically identical vegetative cell (mating) types from one another, are produced by some species of ciliates^{1,2}. Their most striking effect can be observed by exposing cells of one type to a pheromone secreted by another co-specific cell type³. In the presence of this 'non-self' signal, these cells interrupt their vegetative life to unite temporarily in mating pairs. Thus ciliate pheromones have traditionally been associated only with mating induction^{2,4}. However, the identification of autocrine pheromone receptors^{5,6} suggests a broader role, which is supported by the hypothesis that ciliates evolved their mating-type mechanism for pursuing self-recognition¹. We now report studies, in the cosmopolitan marine sand-dwelling protozoan ciliate *Euplotes raikovi*, demonstrating that these molecules promote the vegetative reproduction (mitogenic proliferation or growth) of the same cells from which they originate. As, understandably, such autocrine pheromone activity is primary to that of targeting and inducing a foreign cell to mate (paracrine functions), this finding provides an example of how the original function of a molecule can be obscured during evolution by the acquisition of a new one.

The structural characterization of several members of the apparently unlimited family of pheromones of *E. raikovi* has revealed very little overall sequence identity^{7,8}, although each pheromone is encoded by one of a series of multiple alleles at the same mendelian locus⁹. These molecules do, however, share a well-preserved common architecture, as shown by the determination of their disulphide bond pattern¹⁰, formed between the six half-cystines conserved in the general sequence DX₁₃-CX₆CX₄CX₂₃CX₅₈CX₇₉CX₁₅, and the three-dimensional structure in solution determined by two-dimensional NMR spectroscopy¹¹⁻¹⁴. The principal motif consists of a bundle of three regular, or nearly regular, α -helices with an up-down-up topology. The main pheromone-specific differences reside in the carboxy-terminal region¹⁴.

Because ciliate pheromones have been thought to function only in inducing cell mating, bioassays have so far been carried out with cells in the 'adult' stage of their life cycle that were maximally ready to mate, that is, in an early stage of starvation and thus arrested in their reproduction. To determine whether pheromones of adult *E. raikovi* are uniquely associated with mating, it was first ascertained if cells growing asynchronously in fed cultures—a condition usually not appropriate for mating—also produce pheromones. Cells in asynchronous growth were removed from culture and transferred, at an adjusted initial low density of 200 per ml, into a 5-l volume of fresh medium (artificial sea water) and food (green alga *Dunaliella tertiolecta*). This was added at an amount high enough to allow continuous cell growth during the next 4–5 days. One-litre samples were then removed daily from this new culture and analysed for secreted pheromone according to standard procedures¹⁵. The results showed that growing cells secrete pheromone similar to cells which are in a condition suitable for mating. In fact, 6.2 $\mu\text{g l}^{-1}$ of active protein was purified from

medium removed 24 h after resuspending the cells, and this pheromone concentration increased to 19.5, 50.4 and 114 $\mu\text{g l}^{-1}$ during the next three days, in parallel with a nearly fivefold increase in the cell density.

Evidence that autocrine interactions with the secreted pheromone promote *E. raikovi* reproduction has been based mainly

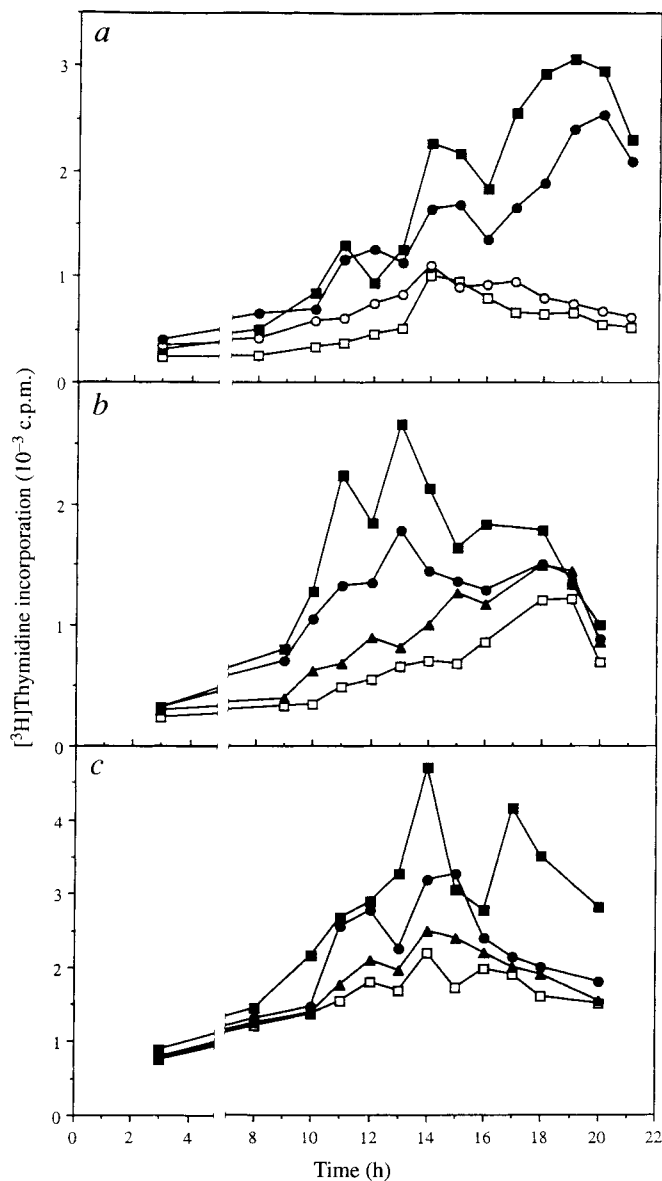


FIG. 1 Pulse analyses of [³H]thymidine incorporation by type I and type II cells suspended with and without their own pheromones. *a*, Cells of type I, secreting pheromone Er-1, and of type II, secreting Er-2, in fresh medium (open squares and open circles, respectively), and preparations of their sterilized filtrates (filled squares and filled circles, respectively). *b*, Type I cells in fresh medium (open squares), and with increasing concentrations of a purified Er-1 preparation, namely 3.4 nM (filled triangles), 34 nM (filled circles) and 340 nM (filled squares). *c*, Type II cells in fresh medium (open squares), and with increasing concentrations of a purified Er-2 preparation, namely 2 nM (filled triangles), 20 nM (filled circles) and 200 nM (filled squares). In these and the following experiments, unless otherwise specified: (i) cells (density, 2,000 per ml) were conditioned, by starvation and time-limited refeeding, to move in partial synchrony from a resting stage into a new division cycle; (ii) the incorporation of [³H]thymidine (specific activity, 15.5 Ci mmol⁻¹) was counted, at the indicated intervals, on 1-ml cell samples incubated for 1 h, collected with a cell-harvester, precipitated and washed with 10% trichloroacetic acid; (iii) values represent the means of three independent measurements taken from replicates of the same experiment.

on comparative analyses of [^3H]thymidine incorporation and cytological observations of the cell-cycle stages, carried out with cells derived from cultures in a resting (G_0) stage, that is, at the end of 3–4 days of starvation and several resuspensions in fresh medium to remove secreted pheromone. These cells were allowed, by a time-limited addition of food, to pass (in partial synchrony) from starvation into a new division cycle (which, at 24 °C, develops through G_1 , S and G_2 -D stages of 6–7 h, 4–5 h and 1 h, respectively), and then examined after resuspension, (i) in the absence of their pheromone, (ii) in sterilized filtrates of their culture medium containing secreted pheromone, and (iii) with varied, physiological concentrations of purified preparations of their own pheromone. As shown in Fig. 1, [^3H]thymidine was always incorporated by cells resuspended in the presence of their pheromone at markedly higher rates than cells in medium initially lacking pheromone; furthermore, the higher the pheromone concentration that was assayed, the higher the incorporation observed. These differences become evident after 8–10 h of resuspension, coinciding, as shown by cytological observations (Fig. 2), with the appearance of the first specimens with a macronucleus (that is, the ciliate somatic nucleus) marked by 'DNA replication bands' that represent the unmistakable indication that a *Euploes* cell is progressing through the S stage of the cell cycle¹⁶.

In these analyses of the cell response to autocrine pheromone interactions, one major practical problem is caused by the continuous secretory activity of the cells: it is virtually impossible to obtain long-term, viable cultures that contain no secreted pheromone. Furthermore, attempts to isolate mutant cell lines defective in pheromone secretion have been unsuccessful owing to the fact that cells apparently unable to secrete their pheromone show a strongly reduced viability and thus cannot be expanded into cultures (unpublished observations).

To circumvent this difficulty, Er-1 pheromone antibodies (Er-1-Ab) were used to precipitate secreted pheromone from cell filtrates containing measured nanomolar pheromone concentrations. Asynchronously growing cells were then suspended in these filtrate preparations in the presence of [^3H]thymidine and the effects of the interruption of the autocrine loop on the cell reproduction were analysed by measuring their incorporation.

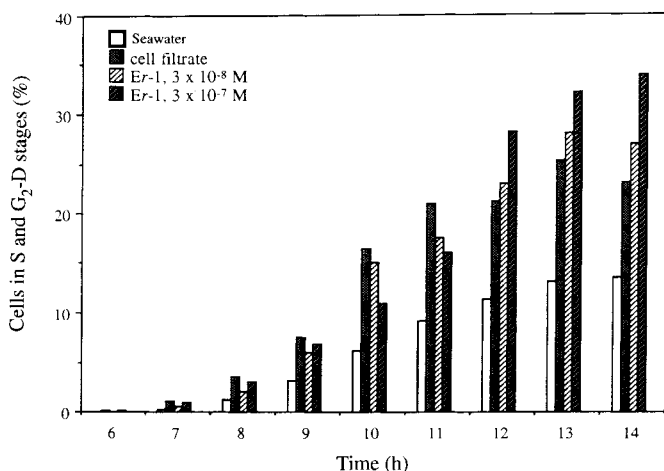


FIG. 2 Cytological analysis of type I cells suspended with and without their own pheromone. At the indicated intervals, cell samples were removed from the suspensions, with and without pheromone (as indicated in the key), and stained with Feulgen reagent for studying their nuclear apparatus. Cells in S and G_2 -D stages were distinguished by the conformation of their macronucleus¹⁶: hook-shaped with DNA replication bands or rod-shaped associated with a mitotic micronucleus, respectively (compared with hook-shaped without DNA replication bands in cells in G_1). Percentages were calculated on at least 300 cells examined from three individual samples.

As shown in Fig. 3a, higher rather than lower incorporation rates were actually found with increasing antibody concentrations.

The structure of the putative *E. raikovi* pheromone receptor-binding moiety^{17,18} explains this activity of the pheromone antibodies well. By an alternative splicing mechanism, each pheromone-secreting cell produces a membrane-anchored pheromone

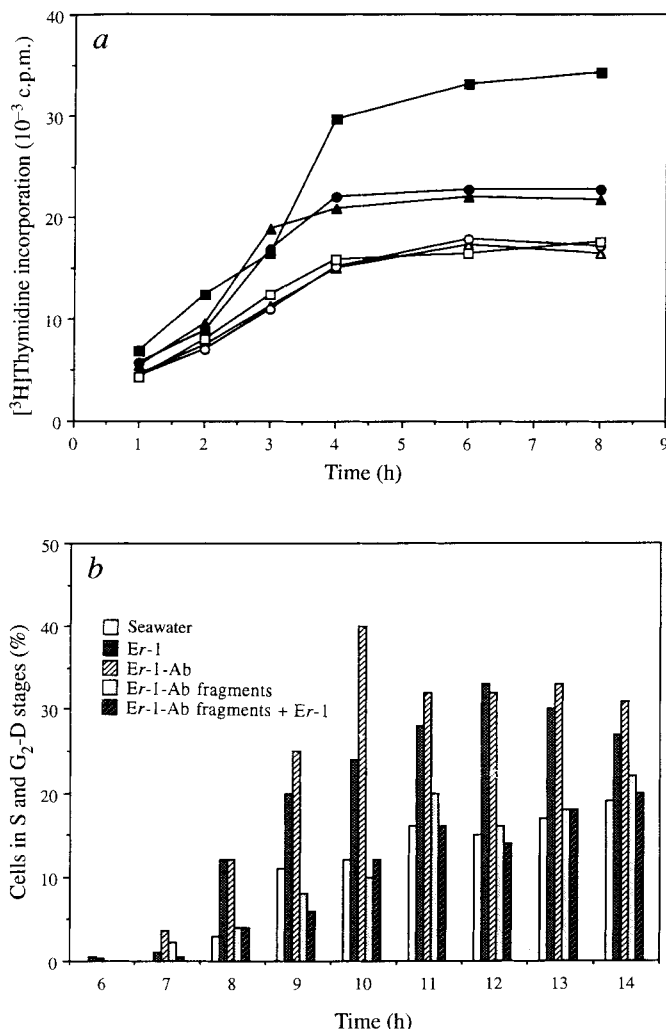


FIG. 3 Effect of Er-1 pheromone antibodies on type-I cell reproduction. a, Time-course analysis of [^3H]thymidine incorporation by asynchronously growing cells suspended with Er-1-Ab. Volumes of cell filtrates containing Er-1 at a concentration of 34 nM were preincubated with Er-1-Ab at different dilutions (1:1,000, filled triangles; 1:500, filled circles; 1:100, filled squares) obtained from an original concentration of 10 mg ml⁻¹. Cells were removed from growing cultures, washed free of food, and resuspended (density 5,000 per ml) in these preparations in the presence of [^3H]thymidine to measure the radionuclide incorporation. No Er-1-Ab addition, open squares, addition of pre-immune serum (100 $\mu\text{g ml}^{-1}$) or bovine serum albumin (100 $\mu\text{g ml}^{-1}$; open circles and open triangles, respectively). Antiserum was prepared as described previously⁵ and its titre measured as 1:8,000 in an enzyme-linked immunosorbent assay. b, Cytological analysis of cells suspended with intact or fragmented Er-1-Ab. At the indicated intervals, cell samples were removed from the suspensions (as indicated in the key) and prepared and analysed as described in Fig. 2 legend. Intact and fragmented Er-1-Ab were used at a concentration of 100 $\mu\text{g ml}^{-1}$ and Er-1 was used at a concentration of 340 nM.

METHODS. Er-1-Ab (5 mg ml⁻¹ in 100 mM sodium acetate, pH 5.5, 30 mM cystine and 1 mM EDTA) were fragmented by partial digestion with papain (insoluble enzyme, 3 mg ml⁻¹). After 12 h at 30 °C, the reaction was stopped by filtering. The filtrate with the Er-1-Ab fragments was dialysed against NaCl (150 mM) and concentrated before use.

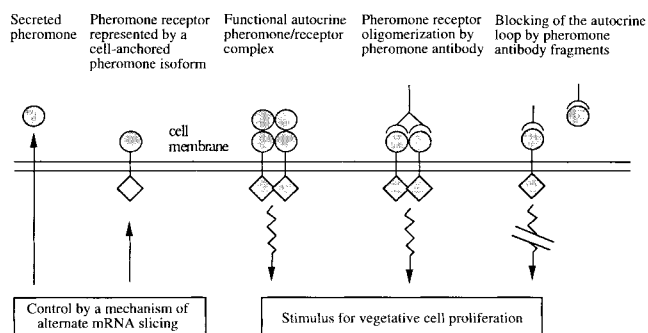


FIG. 4 Diagrammatic representation of the autocrine pheromone-receptor system of *E. raikovi* and its possible interactions with Er-1 pheromone antibodies. The putative pheromone receptor-binding moiety is represented with its extracellular domain identical to that of soluble pheromone as previously determined¹⁷, and in dimeric (active) form in relation with its ligand association according to information principally derived from pheromone crystallographic studies (M. S. Weiss *et al.*, manuscript submitted). A similar phenomenon of dimerization (and activation) is presumed to be induced by the receptor binding to intact Er-1-Ab, and not induced by monovalent Er-1-Ab prepared by digestion with papain.

isoform specified by a messenger RNA that arises from the same primary transcript that yields the pheromone mRNA¹⁷. Because this isoform, orientated in the membrane as a type II protein¹⁹ (with the amino and carboxyl termini lying on the cytoplasmic and extracellular sides, respectively), has the same genetic origin as the secreted pheromone, the amino-acid sequence that forms its extracellular and transmembrane domains is identical with that of the unprocessed pheromone precursor (prepropheromone). Given this context, as shown schematically in Fig. 4, it becomes evident that the pheromone antibodies can bind to the secreted pheromone as well as to its membrane isoforms (receptor-binding moieties), as also reported previously¹⁷, thus potentially causing their clustering and activation by analogy with a number of anti-growth-factor-receptor systems operating in cells of more advanced organisms^{20,21}. That these isoforms can undergo oligomerization on ligand interaction is now documented by the definition of the pheromone association patterns in crystals (M. S. Weiss *et al.*, manuscript submitted), and indirect evidence that this clustering may elicit their activation has been derived from the observation, shown in Fig. 3b, that no stimulatory activity is associated with Er-1-Ab preparations fragmented by digestion with papain, nor with pheromone addition to cells suspended with these preparations.

A mitogenic activity associated with secreted protein has been detected in species of *Dictyostelium*²², *Dileptus*²³ and *Paramecium*^{24,25}. However, the case for autocrine stimulation activity described here for *E. raikovi* pheromones assumes special significance in relation to the cell-type specificity and the pleiotropic, context-dependent effects shown by these molecules. They are in fact the specific messengers of an indefinite number of somatic cell classes (mating types) representing the same organism (species), and apparently elicit distinct (mutually exclusive) biological responses, that is, reproduction and mating, according to whether they bind competitively^{5,26} to cells in an autocrine or paracrine (heterologous) fashion. In an evolutionary perspective, this suggests that the origins of mechanisms that ensure selective cell growth and intercellular collaboration may have preceded the advent of multicellular life. □

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Calcium activation of Ras mediated by neuronal exchange factor Ras-GRF

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TYROSINE kinase receptors stimulate the Ras signalling pathway by enhancing the activity of the SOS nucleotide-exchange factor¹. This occurs, at least in part, by the recruitment of an SOS-GRB2 complex to Ras in the plasma membrane. Here we describe a different signalling pathway to Ras that involves activation of the Ras-GRF exchange factor^{2–4} in response to Ca²⁺ influx. In particular, we show that the ability of Ras-GRF to activate Ras *in vivo* is markedly enhanced by raised Ca²⁺ concentrations. Activation is mediated by calmodulin binding to an IQ motif⁵ in Ras-GRF, because substitutions in conserved amino acids in this motif prevent both calmodulin binding to Ras-GRF and Ras-GRF activation *in vivo*. So far, full-length Ras-GRF has been detected only in brain neurons^{2,6,7}. Our findings implicate Ras-GRF in the regulation of neuronal functions that are influenced by Ca²⁺ signals.

We have previously shown that Ras-GRF does not couple to tyrosine kinase receptors through mechanisms known to operate for the SOS exchange factor⁸. Thus we first investigated the role of Ras-GRF in cell signalling by screening for signals that activate Ras without involving components of the tyrosine kinase cascade, such as Shc proteins. These adaptor proteins become tyrosine-phosphorylated by a variety of tyrosine kinases and then act as docking sites for SOS-GRB2 complexes^{9,10}.

So far, p140 Ras-GRF has been detected only in brain neurons^{2,6,7}. Figure 1a shows that GRF can be detected in primary cultures of newborn rat cortical neurons. We therefore compared two signals that are known to activate Ras in these cells, brain-derived neurotrophic factor (BDNF) and Ca²⁺ influx induced by membrane depolarization¹¹. BDNF was chosen

because it acts through the tyrosine-kinase TrkB receptor, which probably activates the SOS exchange factor. We chose Ca^{2+} influx induced by membrane depolarization because the mechanism by which it activates Ras was unknown. Both stimuli led to significant increases in the proportion of GTP-bound Ras in cortical neurons (Fig. 1b). Shc proteins were then immunoprecipitated from similarly treated cells and immunoblotted with anti-phosphotyrosine antisera (Fig. 1c). As expected, BDNF treatment enhanced the tyrosine phosphorylation of Shc proteins (Fig. 1c). Remarkably, in similar cells treated with KCl there was little if any increase in tyrosine-phosphorylation of Shc (Fig. 1c). In addition, no detectable association of Shc with Grb2 was detected (data not shown). These findings indicated that in these cells calcium could use a distinct non-tyrosine-kinase signalling pathway to Ras that may involve an alternative exchange factor such as Ras-GRF.

The idea that Ras-GRF may mediate Ca^{2+} signalling in neurons was supported by the observation that Ras-GRF contains an IQ motif¹² which allows proteins to bind to calmodulin and/or related Ca^{2+} -binding proteins⁵. Figure 2a shows a linear map of Ras-GRF with the 20-amino-acid IQ sequence which is similar to that in the calmodulin-binding protein neuromodulin. We searched for an interaction between Ras-GRF and calmodulin *in vivo* using epitope-tagged Ras-GRF transiently transfected into 293T cells¹³ that do not express endogenous Ras-GRF. The cells were then stimulated with the Ca^{2+} ionophore ionomycin for 5 min, Ras-GRF was immunoprecipitated, and coprecipitated calmodulin was identified by immunoblotting (Fig. 2b, c). Ras-GRF isolated in this way contained roughly equimolar amounts of bound calmodulin (Fig. 2b, c, lanes 1). In contrast, treatment of immunoprecipitates with EGTA removed associated calmodulin (Fig. 2b, c, lanes 2), indicating that binding was

Ca^{2+} -dependent. This binding activity of Ras-GRF was dependent on an intact IQ motif, because a mutant Ras-GRF with three glutamines substituted for two conserved arginines and a lysine at positions 214, 219 and 223 (Fig. 2a) failed to associate tightly with calmodulin *in vivo* (Fig. 2b, c, lanes 3).

Next we investigated whether Ras-GRF could mediate Ca^{2+} -activation of Ras. A plasmid encoding a (His₆)-tagged RasH protein was transiently transfected into 293T cells with Ras-GRF or the mutant Ras-GRF that failed to bind to calmodulin tightly *in vivo* (Ras-GRF·IQ⁽⁻⁾), or with empty vector. The effect of Ca^{2+} influx on the amount of Ras bound to GTP was then determined (Fig. 3a). In 293T cells lacking Ras-GRF, the baseline state of Ras was ~19% GTP, and this ratio was not altered detectably by ionomycin treatment. In Ras-GRF-expressing cells, however, ionomycin elevated Ras-GTP levels to ~42% from a baseline state of ~32% GTP. Cells expressing the mutant Ras-GRF·IQ⁽⁻⁾ also had raised basal levels of Ras-GTP, but no further increase was observed when the cells were exposed to ionomycin. Wild-type and Ras-GRF·IQ⁽⁻⁾ were expressed to a comparable extent in 293T cells (Fig. 2).

These findings were confirmed and expanded upon by investigating the effect of Ras-GRF on Erk1, a MAP kinase that lies on a signalling pathway downstream of Ras (Fig. 3b). A Myc-epitope-tagged Erk1 was transfected into 293T cells with Ras-GRF, mutant Ras-GRF·IQ⁽⁻⁾, or empty vector. The cells were exposed to ionomycin or epidermal growth factor (EGF), and Erk activity was measured after immunoprecipitation using an *in vitro* kinase assay with myelin basic protein (MBP) as substrate. In cells lacking Ras-GRF, exposure to ionomycin increased Erk activity ~3-fold above levels detected in unstimulated cells. In Ras-GRF-expressing cells, however, Erk-1 activity was about ten times above levels detected in unstimulated Ras-

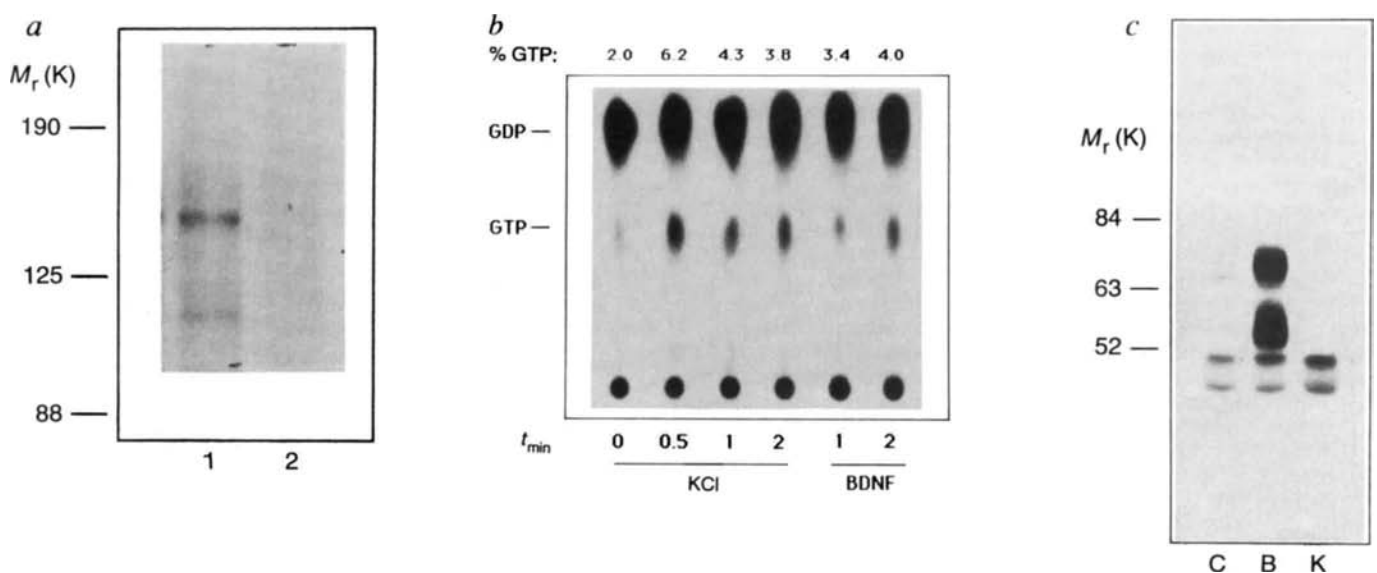
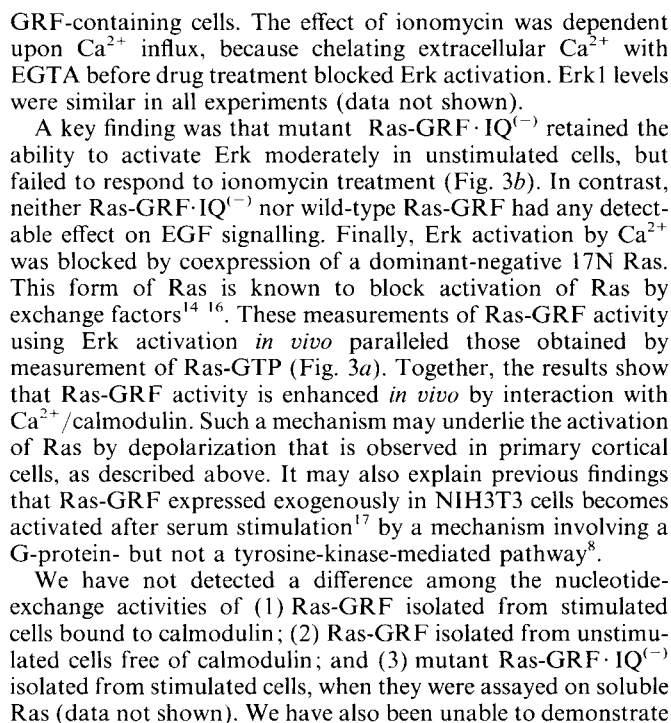


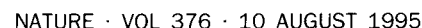
FIG. 1 Activation of Ras but not Shc by membrane depolarization of cortical neurons containing p140 Ras-GRF. **a**, Primary cultures of cerebral cortical neurons derived from newborn rats were metabolically labelled with $^{32}\text{PO}_4$ and then Ras-GRF was immunoprecipitated with either immune serum (lane 1) or immune serum preabsorbed with antigen (lane 2)². **b**, Cortical neurons metabolically labelled with $^{32}\text{PO}_4$ were treated with BDNF (50 ng ml⁻¹) or depolarized by exposure to an isotonic solution containing 50 mM KCl for various times. Ras was then immunoprecipitated from cell lysates and nucleotides bound to Ras were eluted and fractionated by thin-layer chromatography. The migration positions of GDP and GTP standards and the proportion of Ras in the GTP state under each condition (GTP/GTP + GDP) are shown. **c**, Shc proteins were immunoprecipitated (Transduction Laboratories) from cell lysates of untreated neurons or neurons treated with either BDNF for 2 min or raised concentrations of KCl for 1 min. After separation of

proteins by SDS-PAGE, gels were immunoblotted with antisera against phosphotyrosine (4G10-UBI plus PY210; ICN). **C**, control; **B**, BDNF; **K**, KCl. Data are representative of three experiments.

METHODS. Primary cultures of rat cerebral cortical neurons were prepared as described²². Cells were incubated in phosphate-free Dulbecco's modified Eagle's medium for 4 h in the presence of $^{32}\text{PO}_4$ (1.0 mCi ml⁻¹; ICN) before stimulation. For Ras-GRF and Ras immunoprecipitations, cells were lysed in buffer M (20 mM Tris HCl, pH 7.4, containing 150 mM NaCl, 1 mM MgCl₂ and 1% Triton X-100). For Shc precipitation, cells were lysed in HNTG buffer²⁴. For Ras immunoprecipitations, 1 µg ml⁻¹ of anti-Ras antibody Y13-259 was included in the lysis buffer. The proportion of Ras bound to GTP in immunoprecipitates was determined as described²⁵. The per cent GTP was calculated as c.p.m. in GTP/(c.p.m. in GTP + c.p.m. in GDP) after normalizing for moles of phosphate.



METHODS. A modified Glu epitope (MEFMPME) was added to the amino terminus of Ras-GRF by the polymerase chain reaction (PCR) using a 5' primer containing the appropriate codons for these amino acid sequences, plus a *Not*I restriction site 5' and a *Bam*HI site 3' to these codons. This linker was inserted into a clone of Ras-GRF already cloned into the transient expression vector pMT3 (refs 8, 26). The triple mutation changing 3 arginines to glutamines at positions 214, 219 and 223 in the IQ motif of GRF was generated by overlap PCR²⁷. 293T cells (5×10^5 cells in a 60-mm culture dish) were transfected with 1.5 μ g of various forms of GRF in the pMT3 expression vector by calcium phosphate precipitation²⁸, except that DNA uptake by cells was enhanced by allowing precipitated DNA to remain on cells overnight instead of treating cells with glycerol. After cell stimulation, cells were lysed in 0.5 ml buffer M and immunoprecipitated with 20 μ l anti-Glu-epitope monoclonal antibody bound to protein G-Sepharose. Immunoblots were visualized by ECL (Amersham).



a Ca^{2+} -induced increase in the association of Ras-GRF with membranes containing Ras (data not shown). Such a mechanism is thought to occur in the activation of the SOS exchange factor^{18–20} in response to tyrosine-kinase stimulation. Thus, the mechanism of calcium/calmodulin-induced activation of Ras-GRF remains to be determined.

Ras-GRF, through its capacity to activate Ras, may be involved in controlling one or more neuronal processes that are influenced by Ca^{2+} , such as neurotransmitter release, neurite outgrowth, cell survival, cell death, adaptation and potentiation of synaptic transmission²¹. Ca^{2+} entry into neurons occurs through multiple pathways, including a family of voltage-sensitive calcium channels and ionotropic glutamate receptors. Intracellular stores of Ca^{2+} can also be mobilized to increase cytosolic levels. Interestingly, the particular mode of Ca^{2+} entry can dictate a specific cellular response²². Such fine control of Ca^{2+} signalling may be feasible because Ca^{2+} channel types are distributed into distinct subcellular regions, and generate very localized changes in ion concentration²³. Thus, the discovery of which

Ca^{2+} signals activate Ras-GRF, and where Ras-GRF is localized within neurons, may provide clues to its role in influencing neuronal function. □

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◀ FIG. 3 Ionomycin enhances Ras-GRF's ability to activate Ras and Erk1 *in vivo*. **a**, 293T cells were transiently transfected with a 6-histidine-epitope-tagged Ras expression construct together with empty expression vector (C), expression vector containing Ras-GRF, or an expression vector containing mutant Ras-GRF that is unable to bind calmodulin (Ras-GRF·IQ⁽⁻⁾). 48 h later, the cells were serum-starved for 16 h and then metabolically labelled with ³²PO₄ for 4 h. Cells were then incubated with either buffer alone or with buffer containing ionomycin (5 μM) for 5 min, lysed and His-tagged Ras was precipitated from the cells with Ni-agarose. The per cent of Ras bound to labelled GTP (GTP/GTP + GDP) was determined as for Fig. 1. Immunoblot analysis revealed that the levels of wild-type Ras-GRF and Ras-GRF·IQ⁽⁻⁾ mutant in transfected 293T cells were similar (data not shown). The results represent the average of data pooled from three independent experiments, each performed at least in duplicate (±s.e.m.). The difference between Ras-GTP levels in unstimulated and ionomycin-stimulated cells was judged by the Student *t*-test to be significantly different (*P* < 0.001). **b**, 293T cells were transfected with Myc-epitope-tagged Erk1 together with either empty vector (C), vector containing Ras-GRF, vector containing Ras-GRF·IQ⁽⁻⁾, or vector containing Ras-GRF plus dominant-negative 17N Ras. After 48 h, cells were serum-starved for 16 h. They were then stimulated for 5 min with either DMEM alone, DMEM plus ionomycin (5 μM, final concentration) or EGF (10 ng ml⁻¹ final concentration). Erk was then immunoprecipitated from cell lysates and assayed for activity using myelin basic protein as substrate. Data are from one experiment that is representative of three independent experiments. Units of Erk activity were derived arbitrarily from phosphorimager analysis of ³²PO₄ incorporated into myelin basic protein.

METHODS. For **a**, 0.6 μg of pMT3-(His)₆RasH, together with either 3.0 μg pMT3-Ras-GRF or 3.0 μg pMT3 Ras-GRF·IQ⁽⁻⁾, were transfected into 293T cells as described in Fig. 2. Cells were metabolically labelled with ³²PO₄ (0.25 mCi ml⁻¹) in phosphate-free DMEM for 4 h. After stimulation, the cells were lysed in 0.5 ml buffer L (buffer M plus 50 mM NaF, 100 μM Na₃VO₄, 10 μg ml⁻¹ aprotinin, 10 μg ml⁻¹ leupeptin and 1 mM PMSF). After removal of insoluble material by centrifugation at 10,000g for 5 min, the extract was incubated for 45 min with 20 μl of a 50% slurry of Ni-agarose (Qiagen). The beads were then washed 3 times in buffer H (50 mM NaH₂PO₄, pH 8.3, 0.3M NaCl plus 10 μM imidazole). After a final wash in phosphate-buffered saline, nucleotides precipitated with Ras were eluted from the beads and separated by PEI-cellulose TLC as described for Fig. 1. **b**, 3 μg of Myc-Erk1 in pJ3M was transfected together with 3 μg GRF-containing clones. After stimulation, cells were scraped in 200 μl buffer L, centrifuged, and the supernatant incubated at 4 °C for 2 h with 25 μl protein A-Sepharose (50% slurry, Pharmacia) and 10 μl ascites fluid containing anti-Myc antibody 9E10. Beads were washed twice in PBS plus 1% Triton X-100, followed by two washes in kinase buffer (50 mM Tris HCl, pH 7.4, 1 mM DTT, 10 mM MgCl₂). For kinase assay, beads were resuspended in 30 μl kinase buffer containing 25 μM ATP, 0.1 μCi ml⁻¹ [³²P]ATP, 50 μg ml⁻¹ myelin basic protein (Sigma) and incubated at 25 °C for 15 min. After addition of 10 μl 4× Laemmli sample buffer, samples were electrophoresed on a 15% SDS-polyacrylamide gel. Phosphorylated myelin basic protein was identified and quantified with a phosphorimager.

Identification of a specific Ins(1,3,4,5)P₄-binding protein as a member of the GAP1 family

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INOSITOL 1,3,4,5-tetrakisphosphate (Ins(1,3,4,5)P₄) is produced rapidly from inositol 1,4,5-trisphosphate (Ins(1,4,5)P₃) in stimulated cells^{1,2}. Despite extensive experimentation, no clearly defined cellular function has yet been described for this inositol phosphate. Binding sites specific for Ins(1,3,4,5)P₄ have been identified in several tissues^{3,4}, and we have purified one such protein to homogeneity⁵. Its high affinity for Ins(1,3,4,5)P₄, and its exquisite specificity for this isomeric configuration^{5,6}, suggest it may be an Ins(1,3,4,5)P₄ receptor. Here we report the cloning and characterization of this protein as a GTPase-activating protein, specifically a member of the GAP1 family. *In vitro* it shows GAP activity against both Rap and Ras, but only the Ras GAP activity is

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inhibited by phospholipids and is specifically stimulated by Ins(1,3,4,5)P₄.

The open reading frame of the cloned Ins(1,3,4,5)P₄-binding protein is depicted in Fig. 1. The sequence is 60% similar overall to *Drosophila* GAP1 (ref. 7) (72% similar to rat brain GAP1^m (ref. 8)), and from this we conclude that the human circulating-blood clone we have obtained is a member of the GAP1 and GAP1^m family. Because of its Ins(1,3,4,5)P₄ binding we suggest the name GAP1^{IP4BP} to describe this protein. Features of the GAP1^{IP4BP} have been noted for GAP1^m (ref. 8), notably C2A and C2B domains, which, by analogy with synaptotagmin II, are responsible for Ca²⁺-dependent and -independent phospholipid binding respectively (Fig. 1d), a highly conserved Ras GAP domain (Fig. 1c), and a pleckstrin-homology domain⁹. The presence of a pleckstrin domain, which may be involved in plasma-

membrane binding¹⁰, is consistent with our data showing the probable plasma-membrane location of this Ins(1,3,4,5)P₄-binding protein in platelets¹¹.

Purified GAP1^{IP4BP} stimulates GAP activity on Ras with about a fivefold lower potency than p120 Ras GAP, but shows no GAP-stimulating activity at all against Rac or Rab3A (Fig. 2a, b). It proved about equally effective against Rap1A; Maekawa *et al.*¹² reported no detectable activity of GAP1^m on Rap1B. We confirmed that, in our hands, Rap GAP showed no activity at all on H-Ras, and p120 Ras GAP showed no activity at all on Rap1A (Fig. 2b, c) but had a similar specificity profile to GAP1^{IP4BP} against H-, K- and R-Ras (Fig. 2 legend). Although these *in vitro* experiments on purified proteins might therefore suggest that the protein has a novel specificity against Ras and Rap, recent data from budding yeast have shown that

FIG. 1 The amino-acid sequence and domain structure of GAP1^{IP4BP}. a, The nucleotide sequence of the two overlapping clones InsP₄BP-22 and -23, with the predicted amino-acid sequence. Peptides generated from digestion of InsP₄-binding protein purified from porcine platelets are underlined. The primer used to obtain the 5'-nucleotide sequence from clone InsP₄BP-22 is shown boxed. b, Schematic representation of GAP1^{IP4BP}. C2A, C2B, GAP and PH describe the domains with sequence similarity to the Ca²⁺-dependent and Ca²⁺-independent phospholipid-binding domains of synaptotagmin II, GAP-related domain, and PH domain (residues 578–676) respectively. The numbers are residues. c, Sequence similarity among the GAP-related domains of Hs-GAP1^{IP4BP} (residues 274–591), rat brain GAP1^m (Rn-GAP1^m) (D30734; residues 301–619), *Drosophila* GAP1 (Dm-GAP1) (M86655; residues 460–788), human p120 Ras GAP (Hs-p120GAP) (P20936; residues 711–1,020), human neurofibromin (Hs-NF1) (M89914; residues 895–1,166) and human IQGAP (Hs-IQGAP1) (L33073; residues 1,001–1,308). d, Alignment of C2A and C2B motifs among Hs-GAP1^{IP4BP} (residues C2A and C2B 8–112, 140–268 respectively), Rn-GAP1^m (residues 24–138, 166–294), Dm-GAP1 (residues 39–149, 240–438), rat synaptotagmin II (Rn-sytII) (A39454; residues 161–265, 289–412), bovine rabphilin 3a (Bt-rabp-3a) (A48097; residues 421–554, 573–710), Hs-NF1 (residues 2315–2427), and Hs-p120GAP (residues 592–706). A consensus sequence for a C2 domain is shown.

METHODS. GAP1^{IP4BP} was analysed by SDS-PAGE and digested in the excised gel piece with a lysylendoproteinase. Peptides were separated using serial anion exchange and octadecyl reverse-phase columns developed with an acetonitrile gradient. Fractions were applied directly to an automated Applied Biosystems 477A sequencer, modified as described²⁰. Of the 10 peptides sequenced, 5 matched (allowing for frameshifts) with >70% identity the predicted amino-acid sequence of a human complementary DNA termed clone 76 (EMBL A09787), which in itself showed 32% identity with the C-terminal end of *Drosophila* protein GAP1 (ref. 7). Using homologous primers we amplified, by polymerase chain reaction (PCR), a 626-base-pair (bp) fragment from clone 76 (sense 5'-CCGGAGCAGGAGGAGTATCGACGTTTC-3' and antisense 5'-CGTGCACGCAAGACAGACGTGCACGAG-3') by 30 cycles, with one cycle consisting of 1 min at 94 °C, 1 min at 65 °C and 2 min at 72 °C, using a cDNA library reverse-transcribed from a human bone marrow poly(A) messenger RNA (Clontech no. 6573). The PCR product was randomly primed and used to screen a human multiple-tissue northern blot (Clontech); this showed a general widespread tissue expression, with relatively high levels within peripheral blood leukocytes (results not shown). Cloning was performed by screening ~1 × 10⁶ clones of a ZAP Express human circulating-blood cDNA library (Stratagene no. 938202 (oligo(dT) and randomly primed)). Hybridization was performed in QuickHyb containing 1 × 10⁶ d.p.m. per ml ³²P-labelled probe for 12 h at 68 °C. The hybridized filters were washed four times with 0.2 × SSC/0.1% SDS at 60 °C. This procedure yielded, after four rounds of screening, 41 independent clones, which were *in vivo* excised into the pBK-CMV phagmid. One of these clones (InsP₄BP-23) was sequenced on both strands with Exo nuclease III deletion and internal oligonucleotide primers on an Applied Biosystems Automated DNA sequencer (Model 373). To obtain the 5'-terminal sequence an overlapping clone (InsP₄BP-22) was sequenced using an internal oligonucleotide primer that resulted in the identification of a translation initiation codon corresponding well with the Kozak sequence²¹. The open reading frame is of 2,487 bp that encodes a 829 amino-acid protein with a calculated M_r of 94,649. The EMBL accession number is X89399.

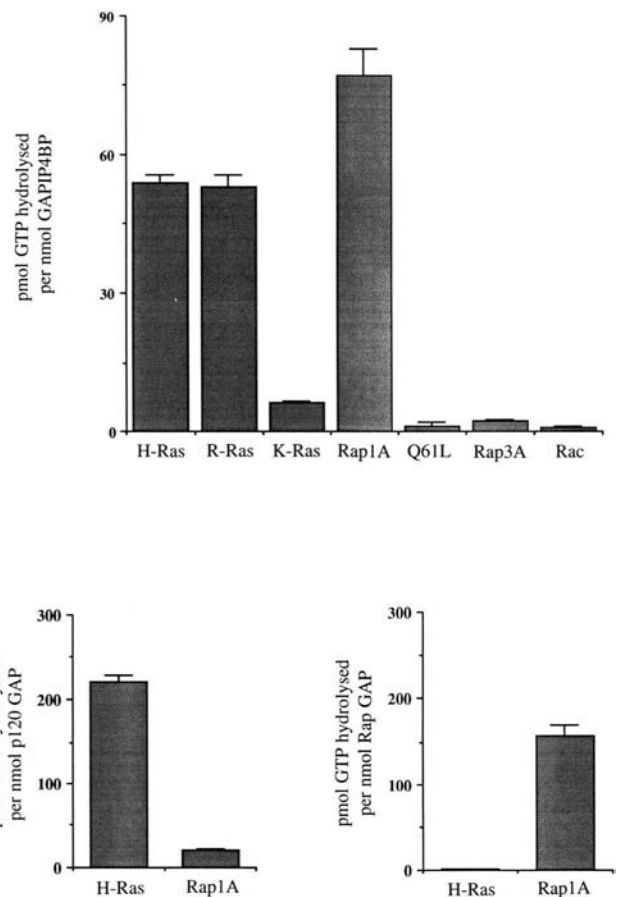


FIG. 2 Specificity of the GAP activity of GAP1^{IP4BP}. a, GAP activity of purified Ins(1,3,4,5)P₄-binding protein on various GTP-binding proteins. b, Selective GAP activity of p120 Ras GAP on H-RAS. c, Selective GAP activity of Rap GAP on Rap1A.

METHODS. GAP assays were performed under first-order kinetics as follows. The required GTP-binding protein (7 µl of 1 µM in 20 mM HEPES (pH 7.3), 2 mM DTT, 1 mg ml⁻¹ BSA) was incubated for 5 min at 25 °C with [³²P]GTP (6,000 Ci mmol⁻¹, Amersham; 7 µl 0.1 µM diluted in 20 mM HEPES (pH 7.3), 2 mM DTT, 1 mM EDTA). Loading was stopped by the addition of 126 µl 20 mM HEPES (pH 7.3), 2 mM MgCl₂, 2 mM DTT. For a single GAP assay, 5 µl of loaded GTP-binding protein was incubated for 10 min with an equal volume of 1 nM GAP (in 20 mM HEPES (pH 7.3), 2 mM MgCl₂, 2 mM DTT, 1 mg ml⁻¹ BSA) at 25 °C. Activity was stopped by addition of 200 µl 5 mM silicotungstate, 1 mM H₂SO₄ with the ³²P being extracted with 300 µl isobutanol/toluene (1/1 v/v); 40 µl 5% (w/v) ammonium molybdate, 2 M H₂SO₄. The upper phase was removed for scintillation counting. Q61L is a p120 Ras GAP-resistant form of Ras. Activities for p120 Ras GAP on H-, R- and K-Ras are 220 ± 8.7, 123 ± 1.7 and 52.8 ± 3.5 pmol GTP hydrolysed per nmol p120 GAP, respectively.

a GTPase with homology to Rap1A is controlled by a GAP that is structurally related to a Ras GAP^{13,14}.

One factor, however, might suggest that Ras is the likely physiological target for GAP1^{IP4BP} (note also that *Drosophila* GAP1 has been proposed⁷ to be a Ras GAP). This lies in the observation that although we found no effect of Ins(1,3,4,5)P₄ on the Ras/Rap GAP activity of the detergent-solubilized protein, we did find that addition of liposomes of a composition similar to that of the inner plasma-membrane leaflet inhibited the GAP activity against Ras but not Rap. The Ras GAP activity was restored by addition of Ins(1,3,4,5)P₄, with a specificity similar to that of displacement of [³²P]Ins(1,3,4,5)P₄ bound to the purified protein^{5,6} (Fig. 3). Another conclusion that emerges from these data is that both Ins(1,3,4,5)P₄-binding and Ras/Rap GAP activity reside within the same protein. This is confirmed by the use of an antibody that we have generated against the carboxy-terminal sequence GDKSFQSYIRQQSETSTHSI. This antibody blotted the Ins(1,3,4,5)P₄-binding protein using both purified protein and crude platelet membranes, and immunoprecipitated from solubilized platelet membranes both high-affinity specific Ins(1,3,4,5)P₄ binding (*K_d* 8.4 ± 1.3 nM), and Ras/Rap GAP activity with a profile similar to the purified protein (results not shown).

The Ins(1,3,4,5)P₄-binding motif can be identified within the C2B domain; this lysine-rich region is responsible for Ins(1,3,4,5)P₄ binding to synaptotagmin II (ref. 15). Note that the lysine residue, identified as important for Ins(1,3,4,5)P₄ binding¹⁵ (asterisk in Fig. 1d), is present in our protein. It is important to note, however, that synaptotagmin II binds InsP₆ with a higher affinity than it does Ins(1,3,4,5)P₄ (with Ins(1,3,4,5,6)P₅ affinity being intermediate)¹⁵, whereas InsP₆ does not bind significantly to GAP1^{IP4BP} (refs 5, 6); we suggest that modification of the nonspecific inositol polyphosphate binding site in synaptotagmin II leads to a highly specific Ins(1,3,4,5)P₄-binding motif. This motif, which includes (R/K)(R/K)TKX(R/K)(R/K)KT, is found in both GAP1^m (ref. 8), and GAP1 (ref. 7), suggesting that these proteins may also be specific Ins(1,3,4,5)P₄-binding proteins (Fig. 1d).

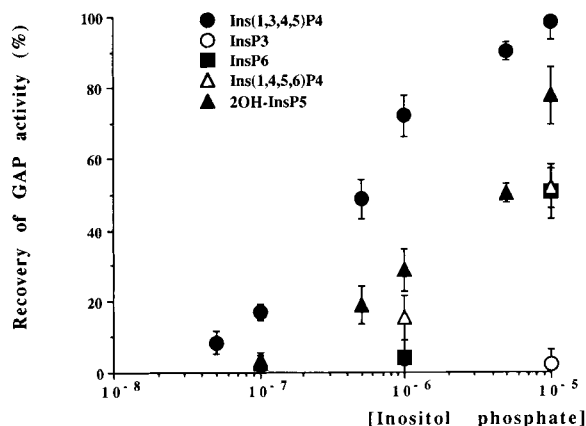


FIG. 3 Inhibition of Ras-GAP^{IP4BP} activity by the presence of inner-plasma-membrane-like liposomes. Specific recovery of Ras-GAP activity by Ins(1,3,4,5)P₄.

METHODS. GAP assays, on heparin peak fractions⁵ (similar results were obtained on purified protein), were performed as described in Fig. 2 with the exception that the following lipids were present (final concentrations); phosphatidylinositol-4,5-bisphosphate (6 µM), phosphatidylinositol-4-phosphate (6 µM), phosphatidylinositol (54 µM), phosphatidylethanolamine (54 µM), phosphatidylcholine (26 µM), phosphatidylserine (53 µM) and sphingomyelin (6 µM). Inclusion of up to 10 µM (5% of total lipid) phosphatidylinositol-3,4,5-trisphosphate (a gift from R. Gigg) had no detectable effect on activity (results not shown). All inositol phosphates are D-isomers, with the exception of D/L Ins(1,4,5,6)P₄. InsP₃ is Ins(1,4,5)P₃.

A connection between phosphoinositidase-C-derived signals and Ras has been suggested in several systems (for example, ref. 16), and the cloning and properties of GAP1^{IP4BP} suggest a mechanism for this. It is also interesting, however, that platelets (anucleate cells) are so rich in GAP1^{IP4BP} and Rap1 (ref. 17), whereas they contain no detectable Ras (ref. 17). Ins(1,3,4,5)P₄, being derived directly (and probably solely) from Ins(1,4,5)P₃, could be argued to have a function directly or indirectly concerned with Ca²⁺ (ref. 1), and there are also, for example, a number of suggestions of links between Ca²⁺ homeostasis at the plasma membrane and Rap1 (ref. 18) or Ras (ref. 19). □

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A human nucleoporin-like protein that specifically interacts with HIV Rev

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THE Rev protein of human immunodeficiency virus type 1 (HIV-1) facilitates the nuclear export of unspliced and partly spliced viral RNAs. Rev contains an RNA binding domain, required for interaction with HIV-1 RNA, and an effector domain, required for RNA-bound Rev to function. The Rev effector domain is believed to interact with a cellular cofactor required for the Rev response and thus HIV-1 replication^{1,2}. Here we report the use of a yeast two-hybrid screen to clone human Rev interacting protein (hRIP), which specifically interacts with the Rev effector domain. This hRIP protein has homology with nucleoporins, a class of proteins that mediate nucleocytoplasmic transport^{3,4}. These and other properties of hRIP are those expected of a Rev cellular cofactor.

The effector domains of HIV-1 Rev and human T-lymphotropic virus (HTLV)-I Rex are functionally equivalent^{5,6}. We

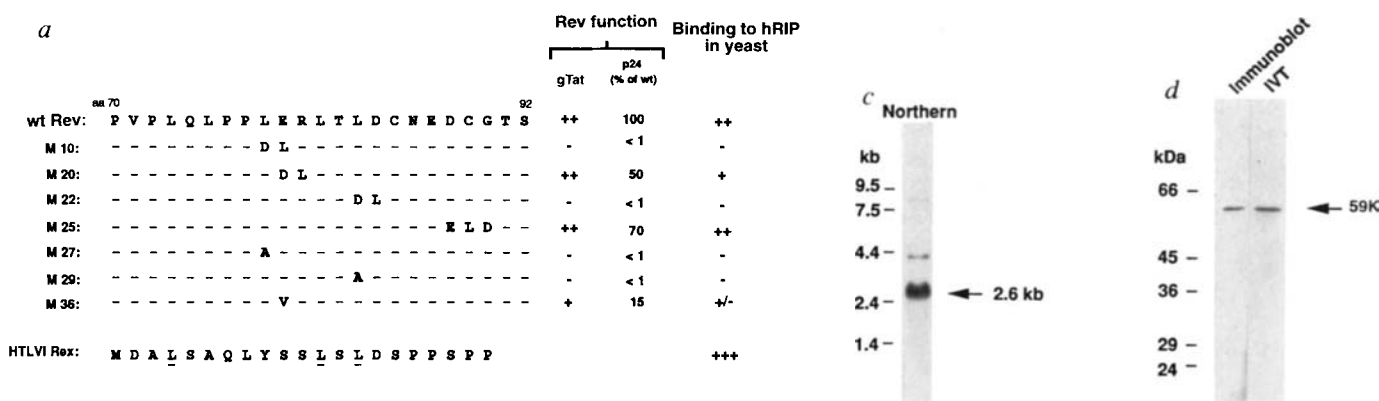


FIG. 1 Identification and isolation of hRIP. **a**, Interaction of hRIP with HIV Rev mutants and HTLV-I Rex. The results of the gTat and p24 assays are from ref. 10. Binding of Rev and Rex derivatives to hRIP was determined in a yeast two-hybrid assay. **b**, hRIP amino acid sequence.

The N-terminal potential zinc-finger is underlined and the relevant cysteines are boxed. In the nucleoporin homology region the FG repeats are boxed and the other repeats underlined. The sequence has been deposited in the EBI/EMBL database, accession no. X89478. **c**, Northern blot. Poly (A)⁺ enriched HeLa RNA (10 µg) was probed with a ³²P-labelled EcoRI-SacI fragment of hRIP. **d**, Immunoblot/*in vitro* translation. An immunoblot using 30 µg HeLa nuclear extract probed with an anti-hRIP antibody (Immunoblot) is compared with a portion of ³⁵S-labelled *in vitro* translation product of the hRIP cDNA (IVT). A polyclonal antibody was raised against an *Escherichia coli*-derived hRIP fragment (amino acids 361–562) and affinity purified against the antigen.

METHODS. Full-length HIV-1-Rev, Rev mutants and HTLV-I-Rex proteins were fused to LexA (1–202) in the vector pEG202 (ref. 8). The two-hybrid screen was performed using an activation domain-tagged HeLa cDNA library⁸ and the LexA-Rex construct as a bait. Two clones were recovered from 1.2 × 10⁷ primary transformants, which both contained the carboxy-terminal portion of hRIP starting at amino acid 361 and 388, respectively. A size-selected human placental cDNA library (ATCC 77399) was screened with an hRIP DNA fragment by standard methods, and two overlapping λ clones were recovered and sequenced.

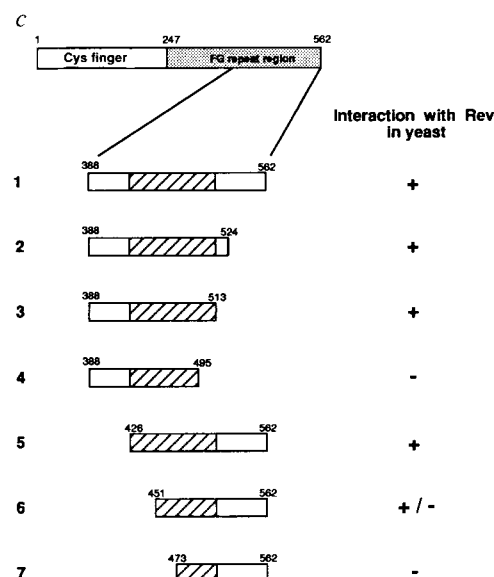
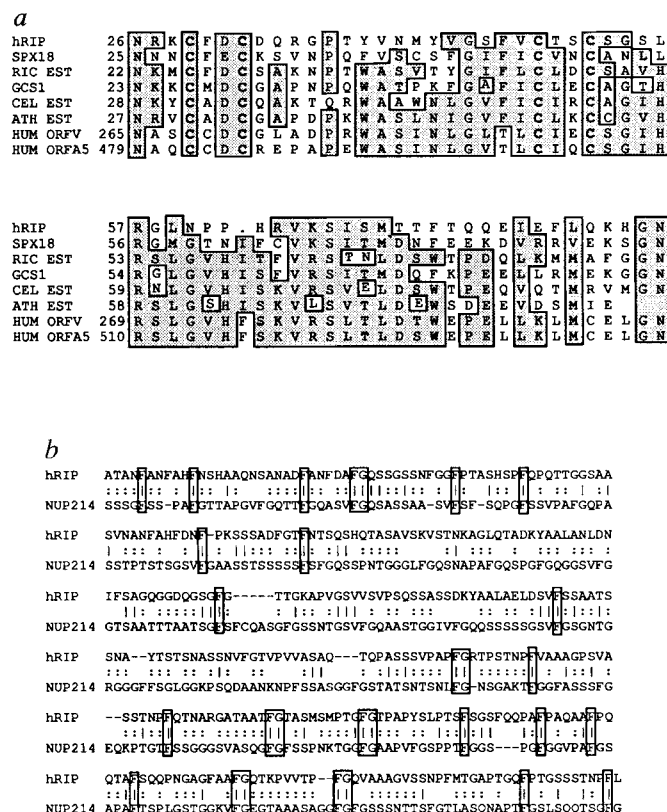


FIG. 2 Sequence homologies and Rev interaction site. **a**, Zinc-finger homology. Proteins in the database with homology to the hRIP potential zinc-finger are shown. Cysteines are bold, conserved residues are boxed and shaded. **b**, Alignment of hRIP and CAN/nup 214. FG repeats and other aligned phenylalanines are boxed. **c**, hRIP region that interacts with Rev. Activation region-tagged versions of hRIP derivatives were tested for interaction with Rev in the yeast two-hybrid assay. The hatched region indicates the minimal boundaries of Rev interaction.

sought to identify proteins that would interact in a yeast two-hybrid assay^{7,8} with both Rex and Rev but not with Rev-M10, which bears a two-amino-acid substitution in the Rev effector domain⁹. We isolated two plasmids that fulfilled these criteria, and contained overlapping complementary DNAs derived from the same gene. The protein encoded by this cDNA was designated human Rev interacting protein (hRIP). LexA fusion proteins of several previously described Rev mutants^{9,10} were tested

for interaction with hRIP in yeast. There was a strong correlation between Rev activity and hRIP binding (Fig. 1a).

The hRIP DNA fragment obtained in the two-hybrid screen was used to isolate a full-length 2.4-kb cDNA containing a 1686-bp open reading frame. The hRIP amino-acid sequence is shown in Fig. 1b. The size of the hRIP cDNA approximates that of the major 2.6-kb band detected in the northern blot of HeLa poly (A)⁺ RNA (Fig. 1c). *In vitro* transcription/translation of the

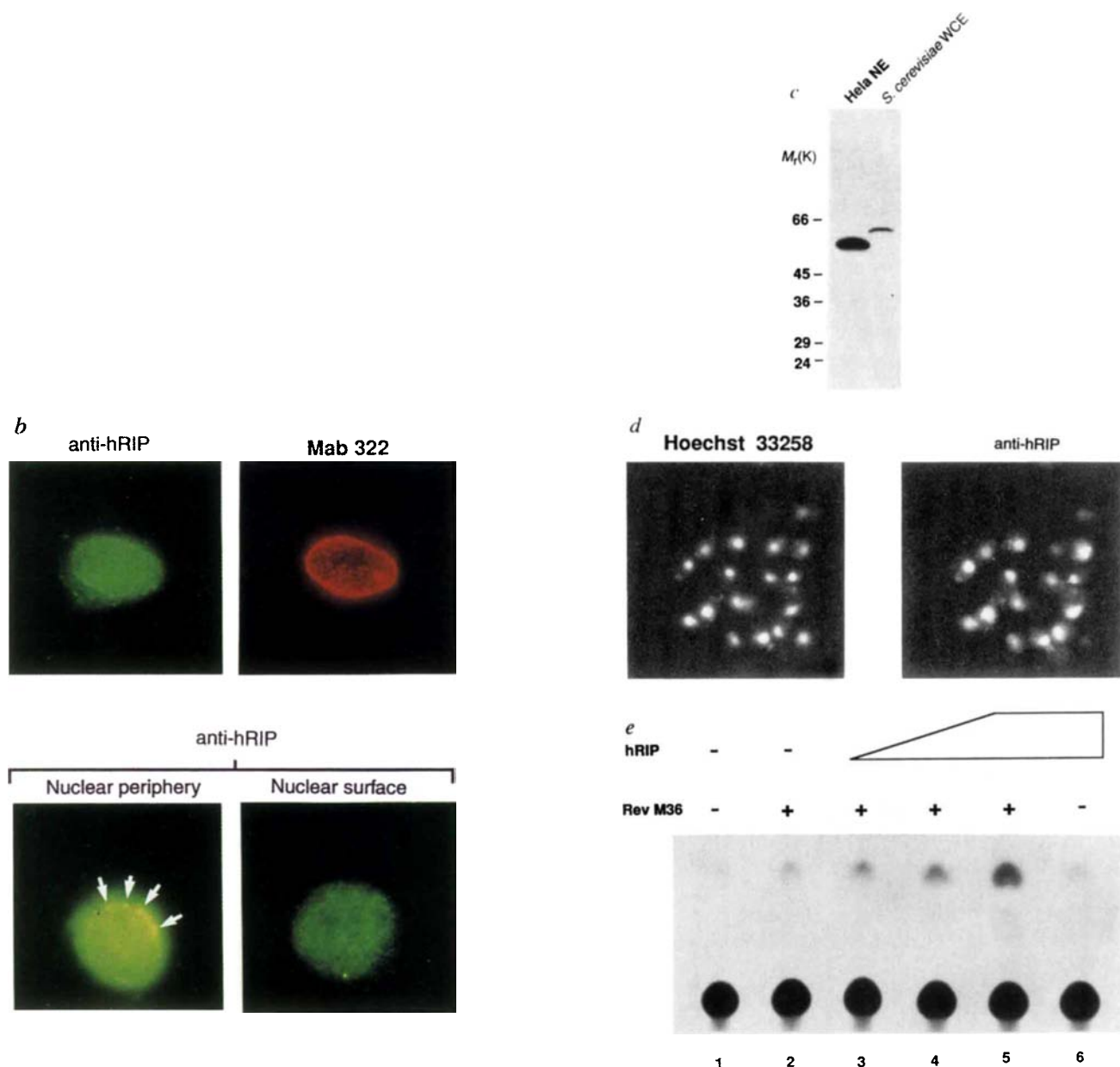


FIG. 3 Intracellular distribution and functional analysis of hRIP. *a*, Distribution of hRIP in fractionated HeLa cells. CE, cytoplasmic extract; NE, nuclear extract; NPE, 7 M urea extract of nuclear pellet¹⁸. Cytoplasmic extract (S100) and nuclear extract were prepared as described¹⁸. *b*, Indirect immunofluorescence of HeLa cells. Mab322 is a monoclonal antibody that recognizes the nucleoporin nup 153 (ref. 19). The punctate stain at the nuclear periphery is indicated by arrows. HeLa cells were fixed for immunofluorescence as described previously²⁰ and probed with anti-hRIP antibody at 7 $\mu\text{g ml}^{-1}$ or Mab322 at a dilution of 1:750. Secondary antibodies: goat anti-rabbit (FITC-TAGO, 1:300) or sheep anti-mouse (cy3-Sigma, 1:500). *c*, Immunoblot analysis of human and yeast cells. NE, nuclear extract; WCE, whole-cell extract. *d*, Indirect

immunofluorescence of yeast cells. Yeast nuclei were made visible by staining of the DNA with the dye Hoechst 33258 (left) and compared to staining with anti-hRIP (right). *e*, Overexpression of hRIP can increase the activity of a Rev effector domain mutant. CAT reporter plasmid pCM128 (0.5 μg) (ref. 18) was cotransfected with the Rev expression plasmid pcRevM36 (0.5 μg) (ref. 10) into CV-1 cells in the absence (—) or increasing amounts (1, 2.5, 5 μg) of an hRIP expression vector. The hRIP expression vector was derived by cloning the full-length hRIP cDNA into pcDNA1 (Invitrogen). Transfection was by the calcium phosphate procedure, and in each case the total amount of pcDNA1 derivative was maintained at 5 μg .

hRIP cDNA resulted in a polypeptide of relative molecular mass 59,000 (M_r 59K), indistinguishable in size from the HeLa polypeptide detected by immunoblotting with an affinity-purified anti-hRIP antibody (Fig. 1d).

Comparison of the hRIP sequence with the databank did not produce a direct match, but several sequence motifs emerged. At the amino terminus, two pairs of cysteines define a subclass of zinc-fingers¹¹ (Fig. 2a). There are also ten XXFG repeats (Fig. 1b), reminiscent of the GLFG and XFXFG repeats found in most yeast and some mammalian nucleoporins³. A yeast nucleoporin specifically involved in messenger RNA export primarily contains XXFG, and not GLFG and XFXFG, repeats¹². One of the highest scores (20% identity, 48% similarity) was achieved with the human CAN protein/nup 214 (ref. 13) (Fig. 2b). hRIP contains several other short repeats (see Fig. 1b), again typical of nucleoporins³. The FG repeats in nucleoporins are believed to mediate protein-protein interactions¹⁴, and Fig. 2c shows that the minimal portion of hRIP required for interaction with Rev is within the nucleoporin homology region.

Virtually all hRIP is present in the nuclear extract of fractionated HeLa cells (Fig. 3a), consistent with results obtained by indirect immunofluorescence (Fig. 3b). Focusing on the nuclear rim (bottom left) or the nuclear surface (bottom right) revealed a punctate staining pattern resembling that of nucleoporins (for examples, see refs 13, 15). However, unlike the typical nucleoporin pattern, most hRIP staining is nucleoplasmic (compare top left and right of Fig. 3b).

Rev can function in *Saccharomyces cerevisiae*¹⁶, suggesting the existence of a yeast homologue of the mammalian Rev cofactor. We probed a yeast whole-cell extract with the affinity-purified anti-hRIP antibody and detected a single yeast polypeptide of M_r 63K (Fig. 3c), very close in size to hRIP (59K). The indirect immunofluorescence experiment (Fig. 3d) shows that the yeast polypeptide, like HeLa hRIP, is nuclear localized. These results suggest that *S. cerevisiae* contains a homologue of hRIP.

To assay for a functional involvement of hRIP in Rev-mediated RNA export we investigated whether overexpression of hRIP could increase the activity of a Rev effector domain mutant. In a mammalian cell cotransfection assay with a chloramphenicol acetyltransferase (CAT) reporter plasmid¹⁷ (pCM128), coexpression of hRIP resulted in a dose-dependent increase in Rev-M36 activity, which normally has 15% Rev function (Fig. 3e, lanes 2–5). hRIP also increased, to a lesser extent, wild-type Rev activity (data not shown). In the absence of Rev, hRIP did not affect expression of pCM128 (Fig. 3e, lane 6), and comparable overexpression of hRIP did not increase expression of cotransfected Rous sarcoma virus (RSV)- and cytomegalovirus (CMV)-promoter-driven reporter genes (data not shown).

Taken together, our results suggest that the interaction between Rev and hRIP is functionally relevant. The sequence features and cellular distribution of hRIP suggest a direct role in nucleocytoplasmic transport. The availability of the hRIP cDNA should facilitate future studies of Rev action, as well as cellular RNA nuclear export. □

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Modulation of enhancer-promoter interactions by insulators in the *Drosophila* embryo

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INSULATOR DNAs, or boundary elements, functionally isolate neighbouring genes by blocking interactions between distal enhancers and inappropriate target promoters^{1–5}. The best-characterized insulators in *Drosophila* correspond to a 340-base-pair (bp) fragment from the gypsy retrotransposon³, and the scs and scs' sequences flanking the 87A1 hsp70 locus^{1,2}. Here we demonstrate that both insulators block the interaction of defined *even-skipped* (*eve*) stripe enhancers^{6,7} when positioned between the enhancer and the target promoter. The simultaneous use of two stripe enhancers (*eve* stripes 2 and 3) provides evidence that enhancers lying distal to the insulator are selectively blocked. The insertion of stripe-insulator-stripe sequences between two divergently transcribed promoters indicates that enhancers barred from acting on one basal promoter are fully accessible to appropriate regulatory factors for activating the other promoter. These results suggest that insulators do not propagate changes in chromatin structure. Finally, we present evidence that the gypsy insulator does not block interactions between a silencer element and a basal promoter. Taken together, these results suggest that insulators might not be restricted to the functional isolation of neighbouring genetic loci. Rather, they might function as flexible regulatory elements that modulate enhancer-promoter interactions within complex promoters and complex genetic loci.

A 340-bp fragment from the gypsy retrotransposon³, containing 12 binding sites for the suppressor of Hairy wing {su(Hw)} zinc-finger protein^{3,5}, was inserted between minimal *eve* stripe 2 and stripe 3 enhancers^{6,7} (Fig. 1). There is a selective loss of stripe 2 expression when the stripe 2 enhancer is placed upstream of the gypsy/su(Hw) insulator fragment (Fig. 1a). Conversely, stripe 3 expression is reduced when the stripe 3 enhancer is placed upstream of the insulator (Fig. 1c). Stripe 7 expression is dynamic and regulated by sequences located within both enhancers, so insulator activity is best assessed by comparing stripes 2 and 3 (see Fig. 1 legend). Evidence that repression is mediated by su(Hw) stems from genetic studies. Expression of the upstream, 'blocked' enhancer is partly restored in embryos containing decreased su(Hw) activity (Fig. 1b, d).

Similar results were obtained with the scs insulator^{1,2} (Fig. 2). Stripe 2 expression is blocked when the stripe 2 enhancer is placed upstream of a 900-bp fragment from the scs insulator (Fig. 2a). This scs fragment mediates a partial block in stripe 3 expression when the order of the enhancers is reversed (Fig. 2b).

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However, the stripe 3 enhancer is completely blocked by the full-length 1.8-kb *scs* insulator (Fig. 2c).

It has been proposed that insulators might mediate directional changes in chromatin structure^{1-4,8}. To determine whether enhancers located upstream of insulators are accessible to regulatory factors, we examined the expression of the *white* marker gene. The P-transformation vector contains two divergently transcribed promoters⁹, the *lacZ* reporter gene is transcribed to the 'right' and the *white* gene is transcribed to the 'left' (as drawn in Figs 1-4). The use of a *white* hybridization probe demonstrates that the minimal *white* promoter responds to the *eve* stripe enhancers in early embryos (Fig. 3a, b). As shown previously, the direct coupling of the stripe 2 and stripe 3 enhancers results in an abnormal staining pattern in which the two stripes are shifted and expanded so that the interstripe region is nearly eliminated⁷ (Fig. 3a). The insertion of a presumed 'neutral' 400-bp spacer sequence from the *twist* promoter¹⁰ ('twi400') restores the interstripe region. This spacer fragment does not function as an insulator, so expression of the distal stripe 2 enhancer is not impeded (Fig. 3b).

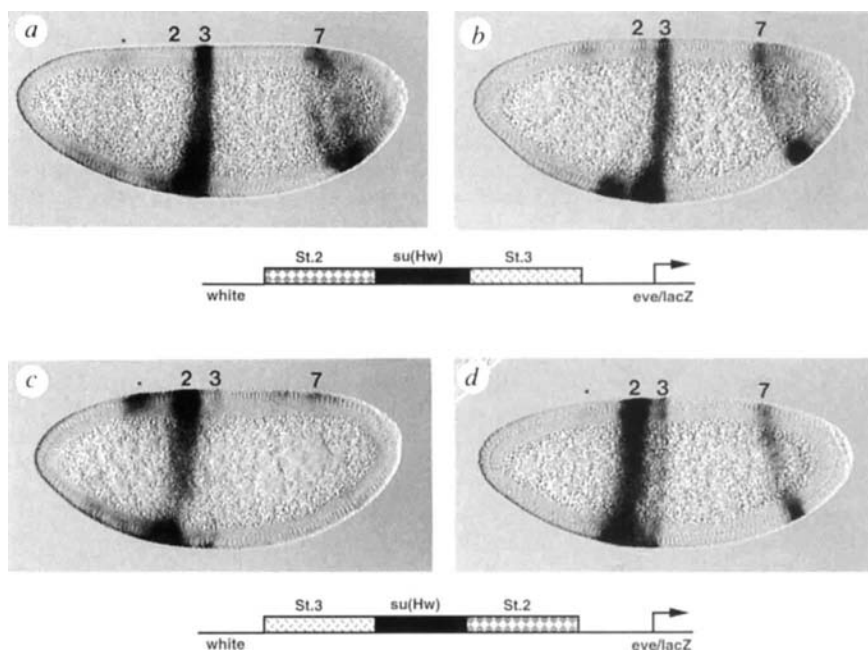
The gypsy/*su(Hw)* insulator leads to a substantial block in stripe 3 expression from the *eve/lacZ* promoter (Fig. 1c), but allows the stripe 3 enhancer to direct robust expression of the *white* promoter (Fig. 3c). The stainings in Figs 1c and 3c are directly comparable because they involved the use of the same transformation vector and transgenic strains. This observation indicates that the insulator does not interfere with the binding of appropriate regulatory factors to the stripe 3 enhancer. The *scs* insulator behaves similarly. It has no effect on stripe 3-*white* expression (Fig. 3d), although it significantly attenuates stripe

3-*lacZ* expression (Fig. 2b). These results provide strong evidence that the gypsy and *scs* insulators do not render distal *cis* elements inaccessible to regulatory factors. Moreover, both insulators lack an intrinsic polarity and block enhancer-promoter interactions regardless of the orientation within the fusion promoters (see diagrams in Figs 1-3).

To determine whether the gypsy insulator can block silencer-promoter interactions, it was placed between the 600-bp *zen* ventral repression element (VR600)^{11,12} and the *eve* stripe 2 enhancer (Fig. 4). As shown previously, the VR600 contains both silencer elements and general activation sequences^{11,13}. Consequently, the VR600-stripe 2 fusion promoter directs an additive pattern of expression, whereby *eve* stripe 2 is repressed in ventral regions (by the dorsal-corepressor silencer), and there is general staining along the dorsal surface beyond the limits of stripe 2 (Fig. 4b). The gypsy insulator results in a selective loss of this general staining (Fig. 4c), so that expression is mediated solely by the *eve* stripe 2 enhancer (compare with Fig. 4a, b). VR600 activation is partly restored in *suHw* mutants (results not shown).

The gypsy insulator does not substantially impede silencing from the VR600 element (Fig. 4c), so that stripe 2 expression continues to be largely excluded from ventral regions. This repression is completely abolished when the fusion gene is expressed in *dl*⁻ mutants (Fig. 4d). The ability of the insulator to block VR600-mediated activation serves as an important internal control, and indicates that the gypsy element selectively blocks activator-promoter interactions compared with silencer-promoter interactions. Nonetheless, it is conceivable that other

FIG. 1 The gypsy/*su(Hw)* insulator blocks distal stripe enhancers. Transgenic embryos expressing fusion genes under the control of the *eve* stripe 2 and stripe 3 enhancers, and containing the 340-bp gypsy/*su(Hw)* insulator (summarized below the panels). Expression patterns were visualized by *in situ* hybridization using a *lacZ* antisense RNA probe labelled with dioxy-genin-uridine^{10,16}. Embryos are oriented with anterior to the left and dorsal up. a, Cellularizing embryo containing the gypsy/*su(Hw)* insulator located between the upstream stripe 2 enhancer and downstream stripe 3 enhancer. The stripe 2 enhancer is repressed, whereas stripe 3 expression is unaffected. Stripe 7 expression depends on regulatory elements located within both enhancers, particularly the stripe 3 enhancer⁷. In addition, the levels of stripe 7 expression depend on the exact stage of development, and the embryos shown in this report vary over an interval of about 30 min (middle to late nuclear cleavage cycle 14). Consequently, insulator activity is best assessed by comparing the expression of stripes 2 and 3. b, As a, except that the fusion gene is expressed in embryos derived from females transheterozygous for a null {*su(Hw)*} and hypomorphic {*su(Hw)*} allele⁵. Stripe 2 expression is partly restored, particularly in ventral regions, owing to the reduction in maternal *su(Hw)* activity. The stronger staining in ventral regions might result from synergistic interactions between bicoid and ventrally localized activators such as twist (D. Arnosti and M.L., unpublished observations). c, Cellularizing embryo containing the gypsy/*su(Hw)* insulator located between the upstream stripe 3 enhancer and downstream stripe 2 enhancer. Stripe 7 staining is abolished, and only a remnant of the stripe 3 pattern can be discerned. In contrast, stripe 2 is expressed at essentially normal levels. d, As c except that the fusion gene is expressed in embryos derived from females carrying *su(Hw)* mutations. Stripes 3 and 7 are partly restored. The transformation vector mediates sporadic staining in head regions (indicated by asterisks in this and all other figures).



METHODS. The 340-bp gypsy sequence was isolated by polymerase chain reaction using *Drosophila* genomic DNA and the following primers: CGGGATCCAATTATTTCG and CAGCGAGCTCGAATTCTATGGCAAGAAA. The identity of the gypsy sequence was confirmed by DNA sequencing. This fragment was inserted between the previously characterized 480-bp stripe 2 enhancer and 500-bp stripe 3 enhancer within the pCasPeR P-transformation vector⁶. Fusion genes were expressed in *su(Hw)* mutants by crossing *white*⁺ males carrying a given P-transposon with virgin females transheterozygous for the *su(Hw)*^v, *bx*^{34e} and *su(Hw)*^f mutations⁵. *white*⁺ male progeny were then backcrossed to transheterozygous virgin females. Embryos were collected and stained for *lacZ* expression.

insulators, or multiple copies of the gypsy insulator, can block the dl silencer.

These findings are consistent with the notion that insulators possess regulatory specificity. First, insulators do not seem to cause long-range changes in chromatin structure that render

enhancers inaccessible to interactions with regulatory factors (Figs 1 and 3). Rather, they appear to function as local 'roadblocks' and consequently should not interfere with the interactions of distal enhancers with distal promoters within complex genetic loci. Second, both the gypsy and scs fragments

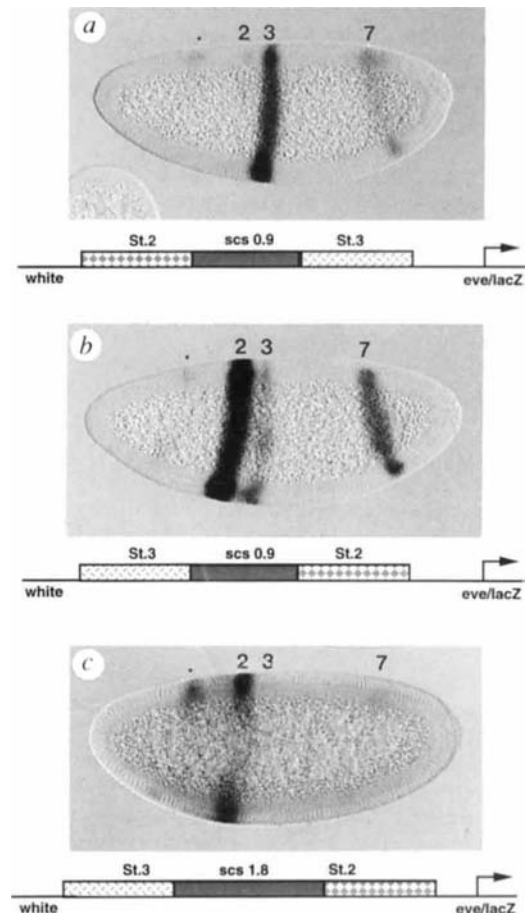
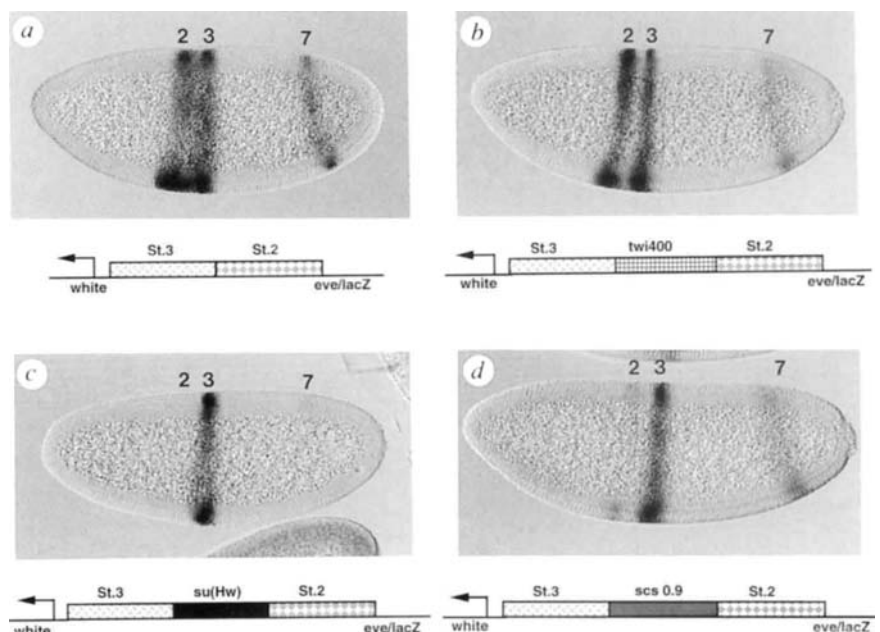


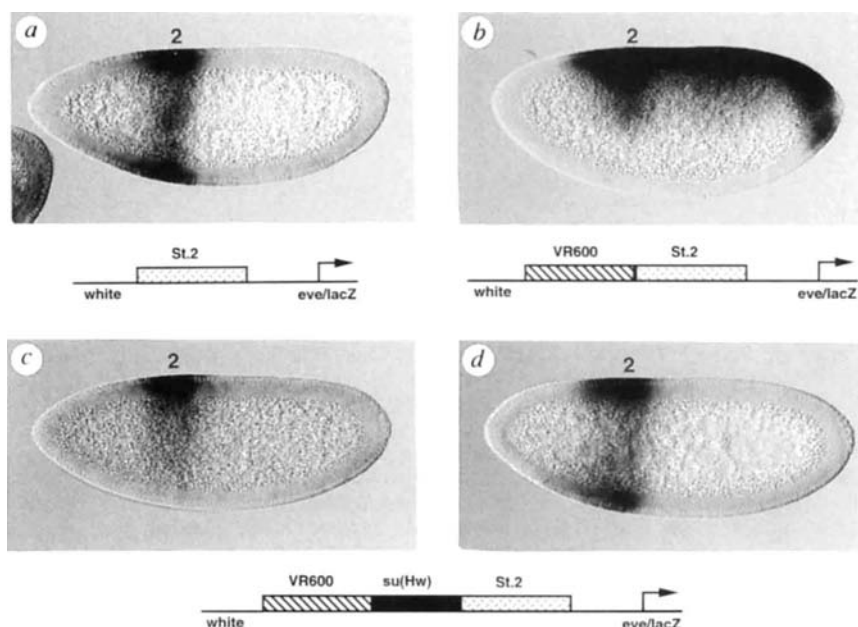
FIG. 2 The scs insulator quantitatively blocks distal stripe enhancers. scs insulator sequences were placed between the eve stripe 2 and stripe 3 enhancers, and expressed in transgenic embryos (Fig. 1 legend). *a*, Mid-nuclear cleavage cycle-14 embryo. Stripe 2-LacZ expression is nearly abolished when the stripe 2 enhancer is positioned upstream of a 900-bp fragment from the scs insulator. The expression of the downstream stripe 3 enhancer is unaffected. Stripe 7 staining is relatively weak owing to the young age of this embryo. *b*, Cellularizing embryo containing the same fusion gene as in *a* except that the orientation of the stripe enhancers was reversed. The upstream stripe 3 enhancer is attenuated, but staining is not fully blocked. In the absence of insulators, stripes 2 and 3 are equally active⁷ (Fig. 3). Stripe 7 staining is relatively strong (compare with *a*) because this is a slightly older embryo. *c*, Mid-nuclear cleavage cycle 14 embryo. Stripe 3-LacZ expression is completely blocked when the stripe 3 enhancer is placed upstream of the full-length, 1.8-kb scs insulator.

FIG. 3 Expression patterns directed by the divergently transcribed white promoter. Transgenic embryos were hybridized with a white antisense RNA probe to detect expression from the 'leftward' white promoter (as drawn below each panel). These embryos are comparable with those presented in Fig. 1, which were monitored for expression of the rightward lacZ promoter. *a*, Cellularizing embryo carrying the eve stripe 3 and stripe 2 enhancers without an intervening spacer or insulator sequence. The two stripes are expressed at equal levels, but there is a loss of the interstripe region. *b*, As *a*, except that a 400-bp 'spacer' sequence from the twist promoter (twi400) was placed between the two stripe enhancers. This twist promoter fragment is located between the distal and proximal elements of that promoter and appears to lack essential cis-regulatory elements¹⁰. The spacer restores a normal expression pattern, consisting of equally intense stripes 2 and 3 separated by an interstripe region. *c*, As *b*, except that the 340-bp gypsy/suHw insulator was placed between the two stripe enhancers. Stripe 3 expression is normal, but the action of the distal stripe 2 enhancer is completely blocked. (It should be noted that the stripe 2 enhancer alone promotes only weak expression of the white promoter, suggesting some type of enhancer-promoter incompatibility. Stripe 2 staining may be strong in *a* and *b* owing to assistance from the neighbouring stripe 3 enhancer.) *d*, As *b* and *c* except that the 900-bp scs insulator was inserted between the two



stripe enhancers. Stripe 3 staining is strong, but expression from the distal stripe 2 enhancer is attenuated.

FIG. 4 The gypsy/su(Hw) insulator does not block silencer-promoter interactions. Transgenic embryos at the early nuclear cleavage cycle 14 stage. All were stained with a *lacZ* RNA probe to detect the expression of the 'rightward' *eve-lacZ* promoter (see diagrams below each panel). **a**, Expression of the *eve* stripe 2-*lacZ* fusion gene. At this early stage the anterior border of stripe 2 has not yet refined. There is equally intense staining in dorsal and ventral regions. **b**, A fusion gene containing the stripe 2 enhancer and upstream 600-bp ventral repression element from the *zen* promoter region (VR600). Previous studies have shown that VR600 contains dorsal and corepressor binding sites that mediate repression in ventral regions of early embryos. In addition, the VR600 contains binding sites for 'ubiquitous' activators that can mediate expression throughout the embryo^{11,12}. The *eve-lacZ* fusion gene is expressed in a composite pattern, owing to the stripe 2 enhancer, as well as both general activators and ventral repressors in the VR600 element. Staining outside the limits of stripe 2 along the dorsal surface is mediated by the general activators in the VR600 element. Staining is completely repressed in ventral regions. **c**, As **b**, except that the 340-bp gypsy/su(Hw) insulator was inserted between the upstream VR600 and the downstream stripe 2 enhancer. The general staining mediated by the VR600 element in dorsal regions (outside the limits of stripe 2) is lost (compare with **b**), indicating that the insulator can block interactions between VR600 activators and the *eve-lacZ* promoter. But silencer activity is not impaired, and stripe 2 continues to



be efficiently repressed in ventral regions. **d**, As **c**, except that the fusion gene is expressed in an embryo derived from *dl+* females. The loss of *dl+* gene activity abolishes the ability of the VR600 element to mediate ventral repression. However, VR600 activators continue to be blocked by the insulator.

quantitatively impede enhancer-promoter interactions (Figs 1 and 2). There is a decrease in gypsy insulator activity as su(Hw) function is reduced (Fig. 1b, d). Similarly, the full-length scs insulator mediates a more complete block in enhancer-promoter interactions than does a subfragment from the insulator (Fig. 2). Third, we observe potential specificity in enhancer-insulator interactions, in that the stripe 2

enhancer is more completely blocked than the stripe 3 enhancer (see, for example, Fig. 2). Finally, the gypsy insulator fails to block the interaction of the dorsal-corepressor silencer^{11,13} with the *eve-lacZ* promoter (Fig. 4). We propose that insulators modulate enhancer-promoter interactions within complex genetic loci such as the Antennapedia¹⁴ and Bithorax¹⁵ complexes. □

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In the stockroom

It's time to clear shelf space for some new biochemical reagents that are now available — synthetic peptide nucleic acids, reagents for nitric oxide synthase research, a fetal bovine serum alternative and freeze-dried guinea pig complement.

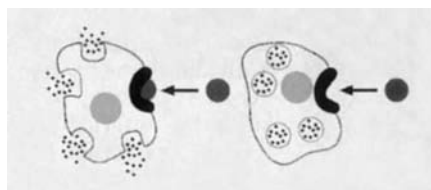
TIB MolBiol offers **synthetic peptide nucleic acids** (PNA) developed by Biosearch (*Reader Service No. 100*). The new DNA analogue consists of the four nucleic acid bases aligned on a polyamine backbone. This allows modifications with FITC and biotin and coupling of peptides. The manufacturer states that PNAs bind their target sequence with greater affinity than comparable DNA oligonucleotides, as witnessed by a drop in their respective T_m (on average 12–15 °C). A longer half-life in cell culture is also to be expected, as no enzyme capable of degrading PNAs has been found *in vivo*. As a consequence of their sensitivity to point mutations in their target sequence, PNAs also make possible the screening of a mixed population of templates for sequence aberrations without tedious sequencing. PNA oligos cannot serve as primers in competition experiments.

Boehringer Mannheim offers the **TriPure isolation reagent** that is designed for isolating RNA, DNA and protein from tissues or cells of human, animal, plant or bacterial origin (*Reader Service No. 101*). This is a monophasic solution composed of phenol and guanidine thiocyanate, and is said to yield 30–150 per cent more RNA than other purification methods. The manufacturer states that this reagent reduces the entire RNA isolation procedure to about one hour. The total RNA is undegraded, free of protein and DNA contamination, and has an $A_{260/280}$ ratio of 1.6 to 2.0. This product should prove useful for Northern blot analysis, RT-PCR, poly(A)⁺ selection, *in vitro* translation, RNase protection assays, cloning or PCR.



Inhibitor tablets.

Pan Vera offers **highly purified, native human Rab3A** for use in signal transduction and protein prenylation research (*Reader Service No. 102*). Rab3A is a member of the Ras-like family of GTP binding proteins and has been shown to be involved in the fusion of exocytotic vesicles with the plasma membrane in neural and pituitary cells. It is a physiological substrate for geranyl-geranyl transferase II — prenylation at the carboxy terminus modulates the interaction with proteins that control the guanine nucleotide-bound state of the protein. The company offers substrates of all three known



Rab3A for signal transduction research.

prenyl transferases: H-ras (WT for farnesyl transferase, H-ras (CVLL) for geranyl-geranyl transferase I and Rab3A. The protein is purified from an overexpressing strain of *E. coli* and is tested for GDP binding and geranyl-geranylation in an *in vitro* assay.

Nitric oxide research

BioMol Research Laboratories now offers **constitutive nitric oxide synthase** (NOS) from a rat brain cDNA isotype (*Reader Service No. 103*). The enzyme is expressed in a baculovirus expression system in the presence of haemin. The manufacturer states that the enzyme is highly purified (>95 per cent by SDS-PAGE) and has high catalytic activity. This product is said to be a useful tool for studying NOS enzyme regulation and screening inhibitors, as well as other applications. A full range of nitric oxide research tools, including NO donors, inhibitors and NOS antibodies, are also available.

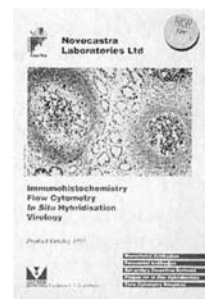
Affiniti Research Products has announced the availability of a range of **reagents for nitric oxide synthase research** (*Reader Service No. 104*). These products are composed of monoclonal and polyclonal antisera to three known isoforms of nitric oxide synthase, and more than 25 biochemicals including precursors, donors, scavengers, cofactors and inhibitors of nitric oxide and its synthases. All of the products are available from stock, with next day delivery throughout Europe. Reagents, such as 3-bromo-7-nitroindazole and *S*-methyl-L-thiocitrulline, are recent additions to the company's line of nitric oxide research products.

Catalogues

Alexis has released its new, **enhanced human plasma and proteins catalogue** (*Reader Service No. 105*). The manufacturer states that this comprehensive catalogue contains innovative reagents needed for researching coagulation, haemostasis, thrombosis and for other research related to blood clotting. It has been designed to bring the researcher information for human plasma enzyme and protein research without many unrelated chemical listings. New products found in the catalogue include annexin V,

β_2 -glycoprotein, factor V, factor VII, factor VIIa, soluble tissue factor, protein C, protein S, protein Z, monoclonal and polyclonal antibodies, as well as many more.

The 1995 **research catalogue** from Novocastra Laboratories is available through Vector Laboratories (*Reader Service No. 106*). It features high-titre monoclonal and polyclonal anti-human antibodies for immunohistochemical, ELISA, western blot and flow cytometry applications, as well as probes for *in situ* hybridization. New products include CD3 monoclonal, effective on paraffin sections. This antibody in combination with the CD20, CD5 and bcl-2 oncoprotein antibodies should play a key role in lymphoma phenotyping. The new Ki67 antibody allows the assessment of proliferation in paraffin-embedded material. Two new fluorescein-labelled probes include CMV and EBV. The company's hormone receptor range has the progesterone receptor monoclonal antibody (NCL-PGR) along with the NCL-ER-LH2 oestrogen receptor antibody.



Novocastra's 1995 catalogue.

The 1995 R&D Systems **product catalogue** contains detailed information on the large range of products the company supplies for immunology, cell biology and molecular biology research (*Reader Service No. 107*). The catalogue is divided into five sections: cytokine proteins and their corresponding antibodies; flow cytometry reagents; adhesion molecule ELISAs and antibodies; Quantikine cytokine ELISAs; and designer genes and probes. These sections also contain detailed reviews of current scientific literature.



1995 immunology, molecular biology and cell biology catalogue.

ELISAs

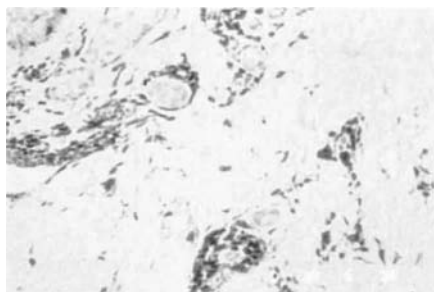
Bender MedSystems has expanded its panel of enzyme-linked immunosorbent assays (ELISAs) for the **quantitative detection of**



Bender MedSystems ELISA for human ICAM-1, -2 and -3.

human adhesion molecules (*Reader Service No. 108*). Besides ELISA kits for the soluble form of intercellular adhesion molecules (ICAM-1, ICAM-2, ICAM-3), selectins (E-, L-, P-selectin) and CD44 standard/variant, the company now offers an ELISA for the detection of soluble platelet endothelial cell adhesion molecule-1 (PECAM-1/CD31). PECAM-1 is a newly characterized adhesion molecule that belongs to the immunoglobulin superfamily. Recently, a soluble form of PECAM-1 has been described in normal human plasma. Increased levels of soluble PECAM-1 in body fluids might be detectable in inflammatory diseases and carcinomas.

Sigma Biosciences has introduced a unique series of **antibodies to S-100 proteins** (*Reader Service No. 109*). Available specificities are monoclonal anti-S-100 (α -subunit, clone SH-A1), monoclonal anti-S-100 (β -subunit, clone SH-B1) and anti-S-100. These products are said to be useful for a wide range of techniques including immunohistochemistry on formalin-fixed, paraffin-embedded tissue, immunoblotting, ELISA and dot blotting. Cross-reactivities



Antibodies to S-100 proteins from Sigma.

include human, mouse, rat, bovine, porcine, rabbit and cat species. The antibodies do not show cross-reactivity with other members of the EF-hand family, such as calmodulin, parvalbumin, intestinal calcium-binding protein and myosin light chain. The antibodies are available in different package sizes (0.2 ml, 0.5 ml and 1 ml). The α subunit of monoclonal anti-S-100 (clone SH-

A1) recognizes the α subunit of S-100a (an $\alpha\alpha$ dimer), anti-S-100 recognizes both α and β subunits of S-100a (an $\alpha\beta$ dimer) and the β subunit of monoclonal anti-S-100 (clone SH-B1) recognizes S-100b (a β monomer).

Growth supplements

BioWhittaker has designed Serum Supreme as an affordable **alternative to fetal bovine serum (FBS)** (*Reader Service No. 110*). The manufacturer states that in tests with four commonly used cell lines, it was

equal to or better than 10 per cent FBS — growth over 12 days, in three of the cell lines was between 40 and 150 per cent higher. The product is manufactured from selected grades of newborn calf serum supplemented with a proprietary mixture of proteins and is said to be capable of replacing FBS in many applications. Its has a low haemoglobin content, which aids in downstream processing and its low levels of endotoxin (<2 EU ml^{-1}), make it an attractive alternative for biotechnology and pharmaceutical production uses.

Don Whitley Scientific has developed **freeze-dried guinea pig complement**, free of azide preservatives and with an improved shelf life, to cover a broad range of applications and serological assays (*Reader Service No. 111*). These include anti-complementary assays for the detection of immune complexes, complement fixation tests and the detection of numerous viruses, such as influenza A and B, Rous sarcoma virus, adenovirus, mumps, measles, herpes simplex virus, and the viruses that cause Q fever and psittacosis, as well as the mycoplasma and *Chlamydia* bacteria. □

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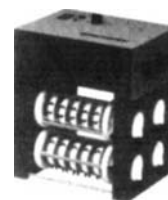
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